Implant- related infections

Diagnostic challenges and insights from an animal model

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DOI (link to publication from Publisher):
10.5278/VBN.PHD.MED.00048

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

IMPLANT-RELATED INFECTIONS

DIAGNOSTIC CHALLENGES AND
INSIGHTS FROM AN ANIMAL MODEL

BY
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DISSERTATION SUBMITTED 2016

AALBORG UNIVERSITY
DENMARK

Implant-related infections: Diagnostic challenges and insights from an animal model

PhD Thesis

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August 2016

Dissertation submitted: August 2016

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PhD Series: Faculty of Engineering and Science,
Aalborg University

ISSN (online): 2246-1248
ISBN (online): 978-87-7112-777-5

Published by:
Aalborg University Press
Skjernvej 4A, 2nd floor
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2016

Preface

This dissertation is submitted in partial fulfilment of the requirements for obtaining the degree of Doctor of Philosophy. The thesis consists of an introduction summarising the literature relevant for the project, based on studies at the crossing point between clinical microbiology, innovative biotechnology, and experimental animal research.

The PhD project was carried out between July 2012 and June 2016 at Department of Clinical Microbiology, Aalborg University Hospital and at the Center for Microbial Communities, Department of Chemistry and Biosciences, Aalborg University. The project was partly funded by a grant for the project “Prosthesis: Reduction of Infection and Pain” (PRIS) from the Danish Agency for Science, Technology, and Innovation. Additionally, Arla Foods Ingredients Group P/S (Denmark) funded part of the *in vivo* study.

First and foremost, I would like to express my deepest gratitude to my two supervisors: Henrik C. Schønheyder and Trine Rolighed Thomsen for giving me the opportunity to work with clinical microbiology in such interesting explorative settings. Thank you Henrik for always dedicating 110% to the work and the interesting discussions of movies not to watch. Thanks, Trine for you unfailing optimism and good discussions, it is fantastic to work with you.

I would like to thank everyone at Department of Clinical Microbiology, Aalborg University Hospital, for creating a good working environment and helping a PhD-student a lot when needed. In particular, Lena Mortensen for training me in the art of culture based clinical microbial diagnostic and covering for me when the PRIS project was running. In Department of Orthopaedic Surgery I would like to

thank the surgeons and especially Vesal Khalid, without whom no patients would have been included.

I would also like to thank everyone in the Environmental Biotechnology group for creating an inspiring environment. My thanks go especially to Yijuan Xu for constructive discussions and great collaboration during the project and to the technicians Jane Ildal, Susanne Beilidt and Marianne Svensson for always being helpful in the lab.

I would like to express my appreciation to the partners in the PRIS project for good collaboration, particularly Jan Lorenzen and Peter Lüttge Jordal at the Danish Technological Institute, and Santina Castriciano, Copan, Italia.

Last but most important I would like to thank the most important persons in my life, Mads and Rosa, for your understanding and patience during the final work-up and for putting the things into perspective.

I hereby declare this is my original work.

Lone Heimann Larsen
August 2016

English summary

More and more people are living with an implant such as pacemakers and joint prostheses. In Denmark the annual number of surgical procedures for insertion of a primary hip or knee prosthesis is approx. 18,000, and the risk of infection following primary implantations is 0.6-2% and higher after revision surgeries. The infection is often located as a bacterial biofilm on the implant or in close proximity to the implant. Consequently, the diagnosis of infection can be a challenge. Biofilm infections are often difficult to diagnose due to the adherence of bacteria to the implant. A surgical intervention is therefore often necessary in order to obtain specimens from relevant sites. A second obstacle is the low growth rate of bacteria in biofilm and the need to 'awake' the persisters, requirements that are not necessarily met by standard culture methods. As a consequence there is a risk of false negative culture reports. By investigating new diagnostic methods, both culture or molecular based, further improvement may be obtained.

The aim of this PhD project was to characterise the clinical diagnostic challenge of prosthetic joint infections (PJI) and to evaluate well-established diagnostic methods together with explorative methods for investigation of the microbial diversity. A third aim was to get insights from a guinea pig *Staphylococcus aureus* biofilm infection model using transcriptomics in order to reveal potential targets for new therapeutic interventions in implant related infections.

The foundation of clinical diagnosis for PJI was discussed, including the contribution of specimen logistic and transport media together with alternative methods for revealing a possible infection: PCR/ESI-TOF-MS (IRIDICA), fluorescence *in situ* hybridization (FISH) and sequencing techniques.
In a large clinical study of prosthesis related problems, the diagnostic of PJI was investigated and the contribution of different specimens obtained in parallel was

evaluated. All specimen types investigated were evaluated both by culturing and 16S *rRNA* sequencing. The optimal specimen set was found to be culturing of a combination of joint fluid, sonication fluid from the prosthesis component, and 5 soft tissue biopsies.

In addition to revealing the infectious organism, the insight from a guinea pig biofilm implant infection model revealed via transcriptomics that the gene expression profile was remarkably similar despite a different course of infection, and that the gene expression profiles also concorded well with a human PJI infection being the source of the strain used for inoculation. The data shows anaerobic pathways to be predominating and pyruvate metabolism in particular. Different strategies were activated in order to handle the acidic environment, and this may be a vantage point for future treatment strategies for *S. aureus* infection.

As a conclusion, the improvement of the diagnosis and treatment of PJI depends foremost on the recognition of the microorganism/s. No single specimen type or diagnostic method has yet been proven to cover the entire spectrum of PJI patients, but by using a specimen collection the coverage improved. Secondly, new treatment strategies are warranted, therefore better understanding of how microorganisms behave in biofilm infections can contribute in that respect. Findings from the guinea pig model revealed remarkable similar gene expression profiles, but different strategies in regulation of stress responses were observed.

Dansk resume

Flere og flere mennesker lever med medicinske implantater som pacemakere og ledproteser. I Danmark bliver der årligt foretaget ca. 18.000 primære hofte- og knæalloplastikker med en risiko for infektion i leddet på 0,6-2% som følge af operationen. Infektionen er oftest lokaliseret som en bakteriel biofilm på protesekomponenterne eller i tæt relation til protesen. Som en konsekvens heraf kan det være svært at identificere den bakterielle biofilminfektion. Et operation er derfor ofte nødvendig for at tage prøver til diagnostik fra relevante steder. En anden udfordring er den lave vækstrate som bakterier i biofilm har fænotypisk; det er ikke optimal i forhold til traditionel dyrkningsbaseret diagnostik, og kan føre til falsk negative dyrkningsresultater. Ved undersøgelse af mulige nye diagnostiske metoder, både dyrkningsafhængige og -uafhængige metoder kan vi måske finde nye strategier til at forbedre den nuværende diagnostik.

Formålet med denne afhandling er at karakterise de diagnostiske udfordringer i klinikken i forbindelse med ledprotese infektioner (PJI) samt at evaluere veletablerede diagnostiske metoder sammen med mere eksplorative metoder med det formål at udforske den mikrobiologiske diversitet hos de inficerede patienter. Ydermere vil en marsvin *Staphylococcus aureus* infektionsmodel med indsatte fremmedlegemer give indblik i bakteriernes metabolisme ved hjælp af transcriptomics og måske afsløre sårbare punkter, som kan bruges i behandlingsøjemed.

De grundlæggende elementer for den klinisk mikrobiologiske diagnose af PJI er diskuteret, inklusivt bidraget til diagnosen fra prøvelogistik og transportmedier samt alternative metoder. De alternative metoder inkluderer PCR/ESI-TOF-MS (IRIDICA), fluorescence *in situ* hybridization (FISH) og sekvenseringsteknikker. I et større klinisk studie af ledprotese-relaterede problemer (PRIS projektet) blev

diagnosen af PJI evalueret, både med standarddiagnostik og udvidet diagnostik med såvel dyrkningsbaserede som dyrkningsuafhængige metoder. Alle prøvetyper blev undersøgt med udvidet dyrkning og 16S *rRNA* sekvensering. Vi fandt, at det optimale diagnostiske prøvesæt til patienter med PJI er ledvæske, protesekomponenterne og 5 vævsbiopsier.

Oveni afslører indblikket fra marsvinemodellen genekspressionsprofiler, som var bemærkelsesværdigt ens for alle infektioner uanset forhistorie, og yderligere viste en sammenligning med en human infektion med samme stamme også dette mønster. Data viser anaerob metabolisme som dominerende og specielt centreret omkring pyruvatomsætningen. Desuden blev påvist flere forskellige strategier betinget af et lavt pH and og andre stress responser. Denne information kan være væsentlig for fremtidige behandlingsstrategier for *S. aureus* infektioner.

Forbedringen af diagnose og behandling af PJI må som første prioritet have afsløring af den inficerende mikroorganisme. Ingen enkeltstående prøvetype eller diagnostisk metode har vist sig at kunne dække hele spekteret af patienter, hvilket understøtter vores anbefaling af et prøvesæt til diagnosen frem for en enkelt prøvetype. Anden prioritet må være udvikling af nye behandlingsstrategier, hvilket kræver et bedre indblik i biofilm infektioner fx fra dyremodeller og analyse af genekspressionsprofiler. Bakteriernes forskellige strategier i forhold til stress responset kan vise sig at rumme nye targets for behandling.

List of papers

Included in the thesis

Paper 1
Larsen, L.H., Lange, J., Xu, Y., and Schønheyder, H.C. (2012). Optimizing culture methods for diagnosis of prosthetic joint infections: a summary of modifications and improvements reported since 1995. J. Med. Microbiol. 61, 309–316.

Paper 2
Larsen, L.H., Xu, Y., Simonsen, O., Pedersen, C., Schønheyder, H.C., Thomsen, T.R., and PRIS Study Group (2014). ”All in a box” a concept for optimizing microbiological diagnostic sampling in prosthetic joint infections. BMC Res. Notes 7, 418.

Paper 3
Larsen, L.H., Khalid, V., Xu, Y., Thomsen, T.R., Schønheyder, H.C. and the PRIS Study Group.
Diagnostic value of culture and 16S r*RNA* sequencing in patients undergoing revision surgery for infection of a hip or knee arthroplasty.
Draft

Paper 4
Larsen, L.H. Xu, Y, Nielsen, K.L., Schønheyder, H.C., and Thomsen, T.R.
In vivo gene expression in a *Staphylococcus aureus* biofilm infection model in guinea pigs: Impact of antibiotic treatment with moxifloxacin.
Draft

Not included papers:
Xu, Y., Maltesen, R.G., **Larsen, L.H.,** Schønheyder, H.C., Nielsen, P.H., Nielsen, J.L., Thomsen, T.R., Nielsen, K.L. (2016). In vivo gene expression in a *Staphylococcus aureus* prosthetic joint infection characterized by RNA sequencing and metabolomics: a pilot study. BMC Microbiology DOI: 10.1186/s12866-016-0695-6

Xu Y., Schønheyder, H.C., **Larsen, L.H.,** Laursen, M. B., Ehrlich, G.D., Lorenzen J., Nielsen, P.H., Thomsen, T.R., and the PRIS Study Group (2014). Characterization of bacterial communities in suspected prosthetic joint infections. In Applications of Molecular Microbiological Methods, Editor: T.L. Skovhus, S.M. Caffrey, and C.R.J. Hubert, eds. Caister Academic Press), pp. 93-103.

Objective of this PhD study

The overall aim of this *PhD project* was to investigate and characterise biofilms in infections associated with prosthetic joints and to study the response to antibiotics in bacterial infection by use of an *in vivo* experimental animal model. The specific goals of the project were:

- To evaluate a highly standardised sampling procedure promoted by pre-packed boxes with all transport media, containers and request forms needed during surgery in patients with a prosthetic joint related problem.

- To identify bacteria by culture-dependent and molecular methods in patients with a prosthesis related problem taking advantage of different specimen types obtained in multiples from the vicinity of the prosthesis following a rigorous protocol and using the pre-packed box.

- To propose an optimal diagnostic specimen set for patients undergoing surgery for a prosthesis related problem based on results obtained with the highly standardised sampling procedure.

- To investigate the gene expression profile of a biofilm infection exposed to antibiotics *in vivo* using a well characterised foreign body animal model.

Abbreviation

CoNS	Coagulase negative *Staphylococcus* spp.
CFU	Colony forming units
CRP	C-Reaktivt Protein
DRNA	Dissimilatory reduction of nitrate to ammonium
EPS	Exopolysaccharide matrix
ESCMID	European Society of Clinical Microbiology and Infectious Diseases
ESI-TOF-MS	High-performance electrospray ionization time-of-flight mass spectrometry
GC-MS	Gas chromatography coupled to mass spectrometry
CR-MS	Capillary electrophoresis coupled to mass spectrometry
ICD	Implanterbar Cardioverter Defibrillator
IDSA	Infectious Diseases Society of America
LC-MS	Liquid chromatography coupled to mass spectrometry
MS	Mass spectrometry
ng	nanogram
NGS	Next generation sequencing
NMR	Nuclear magnetic resonance spectroscopy
OUT	Operational taxonomic unit
PJI	Prosthetic joint infection
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
THA	Total hip arthroplasty
TKA	Total knee arthroplasty
WGS	Whole genome sequencing

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Chapter 1. Prosthetic joint infections

1.1. Background

More and more people are living with an indwelling medical implant such as pacemakers, joint prostheses, and medical pumps. Implants can be an integral part of medical or surgical treatment and may either be temporary or permanent. Additionally, non-medical implants such as fillers are used extensively in cosmetic surgery.

Implant surgery aims to secure or improve the patient's health and life-quality, but despite aseptic precautions and antibiotic prophylaxis during implantations, microbial infection still remains a problem. A complicating infection may have serious consequences for the individual patient, including removal of the implant, but may also lead to significant societal costs.

In the US alone, the hospital costs of hip and knee prosthetic joint infections (PJIs) were estimated to be $556 million in 2009 involving a total of 7,162 hip and 14,802 knee prostheses (Kurtz *et al.*, 2012). Data were drawn from the Agency for Healthcare Quality and Research Register, which cover up to 20% of US hospitals; these estimates did not include costs related to temporary loss of the ability to work. In Denmark statistical data are available for specific types of implants, e.g. hip and knee prostheses and cardiac pacemakers/defibrillators (Danish hip alloplastic register: www.dhr.dk, Danish Knee alloplastic register: www.knee.dk, and Danish pacemaker and ICD register: www.pacemaker.dk). However, a general overview is difficult to find. In the US a top-11 list has been compiled primarily for financial interest (McIntyre, 2011). The annual numbers of hip and knee replacements in this list were 230,000 and 543,000, respectively (Table 1).

In Denmark the annual numbers of primary total hip (THA) and total knee arthroplasties (TKA) were 9,410 (revisions: 1,366) and 8,535 (revisions: 1,291) in 2014 and 2012, respectively (Danish hip alloplastic register, 2015; Danish knee alloplastic register, 2013). The risk of infection after a primary arthroplasty is in the

range of 0.6-2% and higher after revision surgeries (Dale *et al.*, 2012; Gundtoft *et al.*, 2015).

With a steady increase in the numbers of persons with arthroplasties and a continuous rise in life expectancy, more failures due to infection must be foreseen. Thus, a better understanding of PJI is warranted in order to improve diagnosis, treatment, and prevention. The spectrum of microorganisms and their adaption to the foreign body environment are of particular interest. This thesis addresses these issues in relation to hip and knee prosthetic infections.

Table 1. Estimates for the eleven most frequent implants in the US (McIntyre, 2011).

Implants	**Reporting year**	**Numbers**	**Cost (billion $)**
Implantable cardioverter defibrillators (ICD)	2009	133,000	5.5
Hip prostheses	2007	230,000	10.5
Pacemakers	2009	235,000	4.5
Breast implants, purely cosmetic1	2010	366,000	1.0
Spinal fusion hardware	2008	413,000	10
Intrauterine devices	NS	425,000	0.3
Trauma fracture repair	2007	453,000	4.5
Knee prostheses	NS	543,000	12
Coronary stents	2007	560,000	7.5
Tympanostomy tubes	2006	715,000	1-22
Implantable eye lenses (pseudophakos)	2006	2,600,000	8-102

1.2. Infections related to orthopaedic implants

Prosthetic joints are permanent and a 15-year survival rate of 86% with any revision as endpoint has been reported for total hip alloplasties in Denmark

(Mäkelä *et al.*, 2014). The main indications for joint prostheses are arthrosis, femoral neck fracture, and inflammatory arthropathies (e.g., rheumatoid arthritis). The PJI can either occur as a consequence of contamination during surgery or as a consequence of haematogenous spread (Zimmerli, 2014) (for further classification see next section). For both types of infection the prosthesis is colonised by bacteria (rarely by other types of microorganisms) which adhere to the surface and multiply (Zimmerli & Moser, 2012). Some surface materials and areas of the joint prosthesis are more readily colonised than others, e.g., stainless steel has been shown to be more favourable for bacteria than titanium, and the interface between the prosthesis and bone has a higher probability for colonization than the capsule of the joint (Bjerkan *et al.*, 2012; Darouiche, 2001).

For the diagnosis of PJI it is crucial to identify the infecting organism in addition to classify the type of PJI (see next section). Until recently there were no internationally accepted guidelines for the diagnosis of PJI, and therefore either national, regional or even hospital-based criteria have had precedence (Atkins *et al.*, 1998; Zimmerli, 2014). Recently, the Infectious Diseases Society of America (IDSA) and the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) have published guidelines for the diagnosis and treatment of PJI and biofilm infections, respectively (Høiby *et al.*, 2015; Osmon *et al.*, 2013). In addition to clinical findings (elevated temperature, local inflammation and pain), X-ray imaging, and blood biochemistry (sedimentation rate, C-reactive protein (CRP), joint fluid leukocyte count), the focus has been on the microbiological diagnosis. Joint fluid examination and histopathology have been less consistent elements of the diagnosis. Despite the crucial role of identifying causative agents of infection, there has been a lack of thorough studies investigating which specimen types are most reliable on a routine basis. As a consequence, most of the recommendations in the guidelines have been based on expert opinion.

1.2.1. Classification of PJI

PJI has been classified as early (<3months after surgery), delayed (3-24 months after surgery), and late (>2 years after surgery), first by Coventry (1975) and later modified by Fitzgerald and co-workers (Coventry, 1975; Fitzgerald *et al.*, 1977). The classification was based on clinical presentation and level of inflammatory markers, and this was later further elaborated by Romanò and co-workers (Table 2) (Romanò et al., 2011).

Table 2. Spectrum of clinical presentation of PJI (modified from Barrett & Atkins, 2014; Romanò et al., 2011)

Early acute Less than three months after insertion Warm, swollen, painful, erythematous joint ± Features of systemic sepsis	**Early chronic** Less than three months after insertion Persistent wound drainage.
Delayed/late acute More than three months after insertion Warm, swollen, painful, erythematous joint ± Features of systemic sepsis	**Delayed/late chronic** More than three months after insertion Chronic pain ± sinus Loosening may be apparent on X-ray

Together the early PJIs that are typically exogenous, i.e. acquired during implantation or in the period immediately after the surgery via drainage or via the cicatrix with coagulase-negative staphylococci (CoNS) and *Staphylococcus aureus* as the major infectious agents (Stefansdottir *et al.*, 2009). Late PJIs can be caused both by bacteria introduced during implantation or by dissemination through the bloodstream. Infections with a latent period of more than one month are typically caused by microorganisms of low virulence, including CoNS and *Propionibacterium*

acnes (Zimmerli, 2014). Fitzgerald found PJI to be haematogenous in patients presenting symptoms more than 2 years after insertion without previous indications (Fitzgerald *et al.*, 1977) which later are confirmed by a major Swedish study (Stefansdottir *et al.*, 2009). Overall for haematogenous PJIs, *S. aureus* is the most common infectious agent followed by streptococci and Gram-negative bacteria in that order.

Table 3 Bacterial species causing infection in prosthetic joints in hips and knees. (Larsen *et al.*, Draft[Paper 3]; Stefansdottir *et al.*, 2009; Trampuz *et al.*, 2007)

Most comon bacteria in PJI	**Unusual bacteria in PJI**
Staphylococcus aureus	Mycobaterium tuberculosis
Coagulase-Negative Staphylococcie	Peptostreptococcus sp.
Streptococcus sp.	Dermabacter sp.
Enterococcus sp. (facalis/faecium)	Proteus mirabilis
Propionibacteium acnes	Klebsiella pneumonia
Corynebacterium sp.	Haemophilus influenza
Escherichia coli	Peptoniphilus sp.
Pseudomonas aeruginosa	Anaerococcus sp.
Enetrobacter sp.	Aeromonas sp.
Salmonella serovar	Bactoides sp.
Typical bacteria in polymicrobial PJI	
Coagulase-Negative Staphylococcie	
Staphylococcus auerus	
Enterococcus sp.	
Streptococcus sp.	
Finegoldia magna	
Corynebacterium sp.	

The proper classification of joint prosthesis-related infection is important from a therapeutic point of view: Patients with early infection or a likely acute haematogenous PJI with symptoms for less than a month can be treated successfully with 'housecleaning' (implants being retained) and directed antibiotic

treatment, whereas patients with a delayed or chronic infection need to have the implant removed as an adjunct to antibiotic treatment (Zimmerli, 2014).

1.3. Clinical challenges posed by biofilm

The first descriptions of biofilm infections were published in the early 1980s using electron microscopy for visualizing the adherence of bacteria and amorphous material to pacemaker electrodes and intravascular implants (Locci *et al.*, 1981; Marrie *et al.*, 1982). Based on newer research, biofilm infections have been defined by Hall-Stoodley *et al*. as

> ”infections due to aggregated, pathogenic or opportunistic microorganisms encased in an exopolysaccharide matrix (EPS) and recalcitrant to host defence mechanisms and antimicrobial treatment” (Hall-Stoodley *et al.*, 2012).

A previous definition of biofilms required an association with a surface, but the current definition allows e.g., cystic fibrosis to be included. The main characteristic of a biofilm is the aggregation of microorganisms within an extracellular matrix (the EPS described above) composed of self-produced macromolecules including polysaccharides, nucleic acids, proteins, and lipids plus additional host components (Hall-Stoodley & Stoodley, 2009). Biofilms are conceived to be an ancient adaptation of the prokaryotic life form for survival in fluctuating and/or extreme environments and are hypothesised to be the most natural phenotype for bacteria (Hall-Stoodley *et al.*, 2004).

1.3.1. Pathogenesis of prosthesis-related biofilm

The biofilm in a implant-related infection can originate from the human skin, either from the host or the operating staff during surgery or through haematogenous spread (Schierholz & Beuth, 2001). For the biofilm to form, bacteria have to adhere to the surface of the implant, called seeding, where it becomes an irreversible state over time. The surface material, host macromolecules from plasma (e.g., fibrinogen

and fibronectin) and tissues, and the bacterial species and their phenotypes are of major importance for the biofilm formation. In addition, physiochemical properties of the surrounding environment (access to nutrients, pH, and temperature) impact on the growth of the biofilm (Donlan, 2001; von Eiff *et al.*, 2005). After attachment to the surface, changes in the bacterial phenotype begin and a maturation of the biofilm starts.

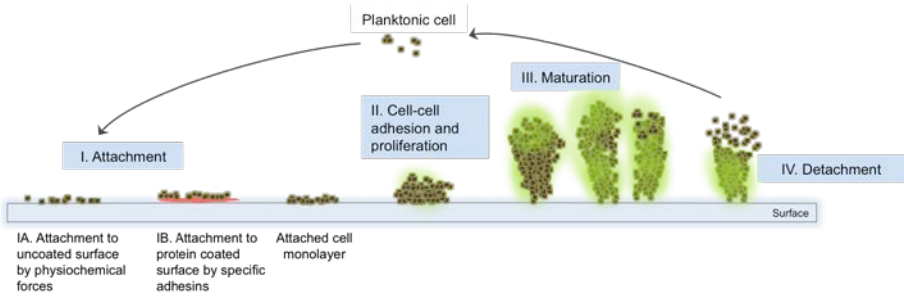


Figure 1 The process of biofilm formations (Xu, 2014, modified Biofilm structer from MSU Center for Biofilm Engineering, P. stoodley & P Dirckx, reprinted with permitision)

Two major benefits of the life in a biofilm are protection against a range of host defences and tolerance towards antibiotics. The EPS matrix restricts the diffusion of effector molecules like immunoglobulins and complement factors, and this is true also for antibiotics. Another protective factor provided by the biofilm is the variation of phenotypes in different zones. In dormant zones the metabolic activity is low, which mitigates against the bactericidal effect of most antibiotics (Stewart, 2002; Walters *et al.*, 2003). Within the biofilm a resistant population of bacteria referred as 'persisters' remain inactive for long periods of time and are the likely explanation for relapses even after long periods of time(Conlon, 2014; Lewis, 2008).

An additional factor explaining the recalcitrance of biofilm against antibiotic therapy is a higher frequency of horizontal gene transfer of resistance and putative

virulence genes in biofilm communities compared with a planktonic population (Costerton *et al.*, 2005; Fux *et al.*, 2005; Madsen *et al.*, 2012)

1.3.2. From the patients to the laboratory and back again

Biofilm infections are often difficult to diagnose due to the adherence of bacteria to the implant. A surgical intervention is therefore often necessary in order to obtain specimens from relevant sites. A second obstacle is the low growth rate of bacteria in biofilm and the need to 'awake' the persisters, requirements that are not necessarily met by standard culture methods. As a consequence there is a risk of false negative culture reports. In order to improve diagnostic accuracy, prosthetic components have been handled aseptically and undergone mild sonication in order to liberate and revive bacteria from biofilms (Gomez & Patel, 2011; Hall-Stoodley *et al.*, 2012). This diagnostic procedure seems to be an adequate response to the challenge posed by biofilms, but by investigating other new diagnostic methods, both culture or molecular based, the diagnostic might be further improved (Larsen *et al.*, Draft)[Paper 3].

Failure to detect biofilm bacteria can have significant consequences (Thomsen *et al.*, 2011). The frequent polymicrobial nature of biofilm can lead to only partial coverage by antibiotic treatment chosen in accordance with results obtained by standard cultures and may even lead to mistaking a PJI for a loosening of the prosthesis. Besides consequences for the individual patient, such mistakes may lead to biased assessment of prosthetic failures overall.

Being a relatively young field of research, many unresolved questions exist regarding the pathogenesis and colonization strategy of the bacteria involved. Werner Zimmerli has pointed out that spontaneously healing of a implant-related infection has never happened (Zimmerli, 2014) – so "knowing the enemy" must be one of the next steps for finding the possible weak spots for a counter-offensive. Experience from the clinical setting and laboratory experiments need to be

supplemented with *in vivo* experiments in order to get more insight into phenotypes and to disentangle the complexity. A crucial point is to analyse effects of alternative antibiofilm compounds on a molecular level, hopefully identifying backdoors for eliminating implant-related infections without a surgical intervention in the future.

1.4. PRIS – a clinical study

"Prosthesis: Reduction of Infection and Pain" (Danish acronym PRIS) was a project running from 2010-2014 with participation of Aalborg University Hospital, Aalborg University, and the Danish Technological Institute together with several private companies. The project was funded by The Danish Council for Technology and Innovation (no. 09-052174). The goals set for the project were to develop new molecular and medico techniques, serological markers, and diagnostic imaging techniques for diagnosis in patients experiencing failure of their hip or knee prosthesis without an immediate cause, i.e. either infection or aseptic failure. The main benefit of the interdisciplinary collaboration should be faster diagnosis and treatment, and avoidance of unnecessary surgical interventions. The PRIS project provided the framework for the extensive microbial diagnostics in Larsen *at al*. (Larsen *et al.*, Draft)[Paper 3].

Chapter 2. Detection and identification of bacteria in PJI

In addition to the findings mentioned in section 1.2, the diagnosis of a PJI is based upon the intraoperative specimens that are instrumental for demonstrating the causative microorganisms and thereby also provides a basis for targeted treatment of infection.

Any diagnostic method must be proven fit for the purpose in accordance with multiple criteria, of which accuracy, robustness and expedience are among the most important (Guyatt *et al.*, 2012; Peeling *et al.*, 2010). Expedient and reliable detection and identification of infecting microorganisms translate into early directed antibiotic therapy. Likewise, timely negative reports have a significant impact on patient management.

2.1. Sampling logistics

> "In every chain of reasoning, the evidence of the last conclusion can be no greater than that of the weakest link of the chain, whatever may be the strength of the rest."
>
> *Thomas Reid: "Essays on the Intellectual Powers of Man" 1786*

In every organization with multiple functions and different locations, logistics becomes a critical issue. The study by Larsen *et al*. (Larsen *et al.*, 2014a)[Paper 2] was influenced by several autonomous factors: It was part of a larger clinical diagnostic study (PRIS) involving several clinical specialities, and patient participation was voluntary for every part of the study. The cooperation was a thorough learning process both for clinical and non-clinical partners, who had to accommodate different aims and practices. This logistic challenge must be kept in mind when considering the design of the study and the inclusion criteria for patients.

2.1.1. Practices of clinical microbiology

In general, staff members of clinical microbiology departments are not directly involved in sampling procedures for diagnostic specimens. In acute care areas, operation theatres and wards, these tasks are taken care of by physicians, nurses, and biotechnicians presumably following specific guidelines.

For a study across different hospitals and with participation of many healthcare workers, a special effort is needed to secure standardised and complete specimen sets. Variation in sampling for standard microbiology has been shown to be critical for sensitivity and specificity of PJI diagnosis (Atkins *et al.*, 1998). Especially for comparisons of specimen types and diagnostic techniques, the individual surgeons' personal skills and preferences would weaken the foundation of the study.

Thus, the primary challenge in our study (Larsen *et al.*, Draft)[Paper 3] was to fulfil an ambitious goal to define an optimal specimen set for the diagnosis of PJI. Many publications have compared two different specimen types against each other (Table 4), but we would like to compare the utility of more than two specimen types and several methods based on the specimens collected simultaneously during the operation. For this purpose a simple strategy was developed (Larsen *et al.*, 2014a)[Paper 3] which made uniform sampling possible across different hospitals and hospital staff. A pre-packed sample box was made, which covered the entire range of specimens taken during surgery and with sample tubes containing appropriate transport medium for every specimen type. The surgeons were informed about the rationale for the multiple specimens, and the colour-coded vials made it relatively simple for a nurse to handle them correctly.

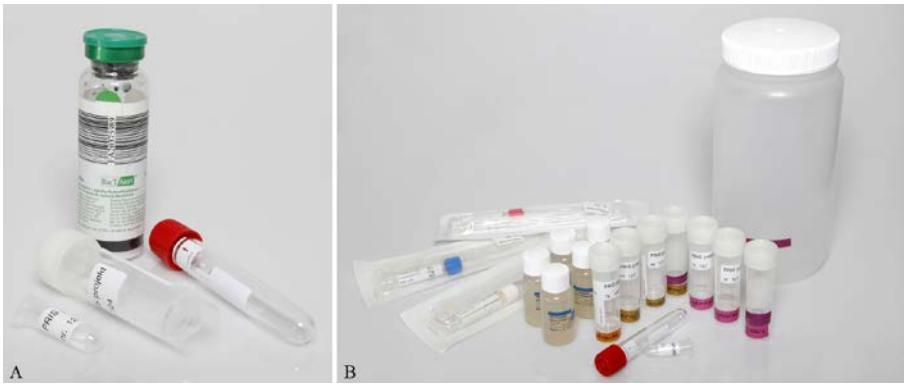


Figure 2**:** (A) Sample boxes for joint puncture and (B) revision surgery. (A) Joint fluid is inoculated directly into a blood culture flask (BacT/Alert, bioMérieux, Marcy l'Etoil, France) and submitted for further culture examination and molecular diagnostics. (B) Sample tubes are colour coded in the revision surgery box in order to assist the operation staff in achieving complete sample sets. Sample tubes had a broad neck in order to facilitate the deposition of the tissue sample (Larsen et al., 2014a)[Paper 3].

Despite its complexity the standardised sampling resulted in a completeness in the order of 90% (updated from Larsen *et al.* 2014a)[Paper 3]. The use of this concept might also be applicable in other areas of surgery and medicine where systematic sampling is important, especially if variation (random or non-random) may impact negatively on the downstream results.

Table 4 Overview of the diagnostic sensitivity and specificity in selected papers, in which it was possible to recalculate the values only for hip and knee PJI. [1]Sensitivity, specificity and 95% confidence interval are calculated by use of www.vassarstats.net, clinical calculator 1.

Reference	Design	PJI diagnosis	No. of patients PJI/Total	Technique	Sensitivity (%, 95% CI)[1] Specificity (%, 95% CI)	Comment
			Synovial fluid			
(Roberts *et al.*, 1992)	Prospective Hip	Culture of tissue biopsy from revison surgery	15/78	Culture (plates)	13/15 (87%, 0.58-0.98) 60/63 (95%, 0.86-0.99)	78 pt had joint puncture followed by revision surgery where one tissue biopsy was sampled for conformation
(Ali *et al.*, 2006)	Prospective Hip	Culture of tissue biopsy from revison surgery	17/73	Blood culture bottel	14/17 (82%, 0.56-0.95) 51/56 (91%, 0.80-0.97)	73 pt had joint puncture followed by revision surgery where ≥5 or 3 tissue biopsies was sampled for conformation
(Trampuz *et al.*, 2007)	Prospective Hip and knee	Visible purulence, acute inflammation, or a sinus tract communicating with the prosthesis	79/331	Culture (plates+liquid) and Blood culture bottels	18/32 (56%, 0.38-0.73) 2/108 (98%, 0.93-1.00)	Only a subset of patients had synovial fluid taken
(Gomez *et al.*, 2012)	Prospective Hip and knee	Visible purulence, acute inflammation, or a sinus tract communicating with the prosthesis	135/366	Blood culture bottel (if more than 1 ml, culture on agar plates)	55/135 (41%, 0.32-0.50) 4/231 (98%, 0.95-0.99)	Combination of sonication fluid culture and PCR had a sensitivity of 78,5% and specificity of 97% The sensitivity are not the same as in the original manuscript (64.7%)
(Cazanave *et al.*, 2013)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	144/434	Blood culture bottel (if more than 1 ml, culture on agar plates)	59/89 (66%, 0.55-0.76) 5/161 (97%, 0.92-0.99)	Only a subset of patients had synovial fluid taken
(Ryu *et al.*, 2014)	Retrospective knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	64/95	Blood culture bottel (if more than 1 ml, culture on agar plates)	44/61 (72%, 0.59-0.82) 1/28 (96%, 0.80-1.00)	Not all patient had synovial fluid taken. A sub group of patient are included in Cazanave et al. 2013 study, where additional diagnostic were preformed on sonication fluid

Reference	Design	PJI diagnosis	No. of patients PJI/Total	Technique	Sensitivity (%, 95% CI)[1] Specificity (%, 95% CI)	Comment
			Tissue biopsies			
(Trampuz *et al.*, 2007)	Prospective Hip and knee	Visible purulence, acute inflammation, or a sinus tract communicating with the prosthesis	79/331	Culture (plates+liquid) and Blood culture bottels	2 positive specimen 48/79 (61%, 0.49-0.71) 2/252 (99%, 0.97-1.00)	Unknown number of interoperative tissue biopsies collected The sensitivity and specificity were caculated for the diferences of 1 or 2 positive speciment
(Achermann *et al.*, 2010)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	31/41	Culture (plates)	19/31 (60%, 0.42-0.78) 0/10 (100%, 0.66-1.00)	Total patient inclute 10 selected negative controls (aseptic failure)
(Gomez *et al.*, 2012)	Prospective Hip and knee	Visible purulence, acute inflammation, or a sinus tract communicating with the prosthesis	135/366	Culture (plates)	95/135 (70%, 0.62-0.78) 3/231 (99%, 0.96-1.00)	Combination of sonication fluid culture and PCR had a sensitivity of 78,5% and specificity of 97%
(Portillo *et al.*, 2012)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	21/81	Culture (plates)	16/21 (76%, 0.52-0.90) 0/60 (100%, 0.92-1.00)	Only hip and knee prosthesis are included in the table. The numbers are based on table 2 & 6 in the manuscript The calculated sensitivity for culturing of tissue biopsies in the paper does not correlate with the data in table 6 (67% vs. 79% (New))
(Cazanave *et al.*, 2013)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	144/434	Culture (plates+liquid)	101/144 (70%, 0.62-0.77) 6/290 (98%, 0,95-0.99)	
(Ryu *et al.*, 2014)	Retrospective knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased	64/95	Culture (plates+liquid) Multiplex PCR	44/64 (68%, 0.56-0.79) 0/31 (100%, 0.86-1.00) 10/64 (16%, 0.08-0.27)	A sub group of patient are included in Cazanave et al. 2013 study, where additional diagnostic were preformed on

Reference	Design	PJI diagnosis	No. of patients PJI/Total	Technique	Sensitivity (%, 95% CI)[1] Specificity (%, 95% CI)	Comment
		synovial fluid leukocyte count			1/31 (97%, 0.81-1.00)	sonication fluid
			Sonication fluid			
(Trampuz *et al.*, 2007)	Prospective Hip and knee	Visible purulence, acute inflammation, or a sinus tract communicating with the prosthesis	79/331	Culture (plates+liquid) and Blood culture bottels)	62/79 (78%, 0.68-0.87) 3/252 (99%, 0.96-1.00)	5 CFU pr culture plate was used as a cut-off on the culture plates.
(Achermann *et al.*, 2010)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	31/41	Culture (plates) Multiplex PCR	17/31 (55%, 0.36-0.72) 0/10 (100%, 0.66-1.00) 26/31 (84%, 0.66-0.94) 0/10 (100%, 0.66-1.00)	Total patient inclute 10 selected negative controls (aseptic failure)
(Gomez *et al.*, 2012)	Prospective Hip and knee	Visible purulence, acute inflammation, or a sinus tract communicating with the prosthesis	135/366	Culture (plates) Multiplex PCR	98/135 (73%, 0.64-0.80) 4/231 (98%, 0.96-0.99) 95/135 (70%, 0.62-0.78) 5/231 (98%, 0.95-0.99)	Combination of sonication fluid culture and PCR had a sensitivity of 78,5% and specificity of 97%
(Portillo *et al.*, 2012)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	21/81	Culture (plates) Multiplex PCR	16/21 (76%, 0.52-0.91) 1/60 (98%, 0.90-1.00) 20/21 (95%, 0.74-1.00) 0/60 (100%, 0.93-1.00)	Only hip and knee prosthesis are included in the table. The numbers are based on table 2 & 6 in the manuscript
(Cazanave *et al.*, 2013)	Prospective Hip and knee	Visible purulence, acute inflammation, sinus tract communicating with the prosthesis or a increased synovial fluid leukocyte count	144/434	Culture (plates) Multiplex PCR)	105/144 (73%, 0.65-0.80) 5/290 (98%, 0.96-0.99) 111/144 (77%, 0.69-0.83) 6/290 (98%, 0.95-0.99)	

2.1.2. Transport media

Ideally a diagnostic specimen should be processed immediately after sampling, but there are numerous barriers to such a level of service even in a research setting, and it would also hamper the translation of the results into clinical practice especially if highly skilled personal is required out of hours. Therefore, the next best option is to secure transport of specimens to the laboratory in their original condition as soon as possible by existing transport services. For the purpose of culturing, microorganisms have to be kept alive outside of the human body and with the composition of microbial communities being as intact as possible. Therefore, the choice of transport media requires knowledge of the likely microorganisms and downstream analysis together with practical considerations in the clinical situation.

For culture-based diagnosis of PJI, the transport media have to preserve a broad range of skin commensals including anaerobes and fastidious bacteria. Three widely used transport media are available on the marked, Amies (Amies, 1967), a variation thereof, and Stuart medium (Stuart, 1946). These media have been evaluated according to the M40-A protocol (Clinical and Laboratory Standard Institute, USA) by Tano and Melhus (Tano & Melhus, 2011). After 24 hours of simulated transport at room temperature all media were able to maintain bacterial viability, but the Stuart medium had the lowest ability to maintain the number of colony forming units (CFU) per mL whereas Amies medium promoted growth that might be a problem when tested with a polymicrobial inoculum. A drawback with the study of Tano and Melhus is the limited selection of bacteria tested. In recent years the focus has mainly been on the evaluation of swab transport systems in relation to the physical properties of the swab while the media have been variations of those mentioned above (Stoner *et al.*, 2008).

The choice of transport media for molecular testing is strongly dependent on the downstream analysis. As an example, in the PRIS study the extraction of nucleic

acid for 16S *rRNA* sequencing analysis was done by use of MolYsis complete 5 kit (Molzym, Germany), which removes human DNA and extracellular DNA before the extraction of the bacterial DNA from intact cells. Therefore it was of great importance to secure that bacteria in the specimens were preserved intact during long-term storage, which was fulfilled by a transport medium with a final concentration of >10% glycerol (Larsen *et al.*, 2014a)[Paper 3].

2.2. Diagnostic methods for PJI in clinical microbiology

At the time of conception of the PRIS protocol there was no international gold standard for the diagnosis of PJI. Nevertheless, culturing tissue biopsies obtained from the vicinity of the prosthesis was given high diagnostic weight. The recently published guidelines from ESCMID and IDSA (see page 19) both conclude that there is no unambiguous definition of PJI, but both recommend the use of multiple intraoperative tissue biopsies for the microbial diagnosis and processing of components of the prosthesis is mentioned only as a possibility (Høiby *et al.*, 2015; Osmon *et al.*, 2013).

2.2.1. Diagnostic samples for microbiology

Since the mid 1990's orthopaedic surgeons in North Denmark Region have used 5 soft tissue biopsies obtained according to Kamme & Lindberg for diagnosis of PJI. The biopsies are sampled from one location in proximity of the prosthesis with separate surgical instruments, sent in separate containers, and processed individually (Kamme & Lindberg, 1981; Mikkelsen *et al.*, 2006). In the original study, Kamme & Lindberg obtained specimens both during primary total hip arthroplasties in patients without signs of infection and during revision surgery in patients with infectious and non-infectious loosening of the prosthesis. A cut-off of ≥3 biopsies with concordant culture findings made the best distinction between bacteria of clinical significance and contaminants.

The systematic use of multiple specimens from the area possibly infected reduces the risk of a false positive diagnosis of infection as many infections are caused by

normal skin commensals that may contaminate the surgical field or culture media in the laboratory, such as *P. acnes* and *Staphylococcus epidermidis* (Osmon *et al.*, 2013). During the last 20 years the importance of multiple specimens have been corroborated (Atkins *et al.*, 1998; DeHaan *et al.*, 2013; Hall-Stoodley *et al.*, 2012). Recently, Bémer and co-workers confirmed the need for multiple specimens and in addition they introduced an optimal number of four independent of the specimen types, thereby acknowledging the legitimacy of reducing resources needed for diagnosis of PJI as much as possible (Bémer *et al.*, 2015).

Culturing is often used as the gold standard for diagnosis of bacterial infections. It is a relatively simple method and only standard equipment is necessary, but skilled laboratory technicians are required for correct handling. The detection level can be very low in terms of CFU and is relatively robust to variation in the ratio between bacteria and human tissue cells. Conversely, prolonged incubation and multiple inspections of bacteriological media are time consuming, and despite of all efforts some viable bacteria may not be able to grow, and previous antibiotic treatment may lead to spuriously negative culture results. The principles of culture methods in clinical microbiology were originally invented in the laboratory of Robert Koch and were aimed to suit planktonic bacteria (Löffler, 2001). Reduced effectiveness for bacteria growing in biofilms has been demonstrated (Hall-Stoodley *et al.*, 2012). The main explanations given for culture-negative PJI are inaccurate sampling, antibiotic treatment before sampling, infection caused by fastidious or unculturable bacteria, insufficient incubation time, and biofilm mode of growth (Hall-Stoodley *et al.*, 2012; Parvizi & Harwin, 2014; Zimmerli *et al.*, 2004). Therefore, alternative methods and specimen types have been investigated in a number of studies comparing sensitivity and specificity (Table 4).

In an early phase of the PRIS study, Xu and co-workers (Xu *et al.*, 2012) showed that molecular techniques could reveal microorganisms in addition to those detected by standard culture methods. Several explanations for the discrepancies were considered: a too brief incubation period for culturable bacteria, delayed

switch from 'biofilm mode of growth', or unsuited culture media. Still, some of the bacterial species found only by molecular methods should be straightforward to culture, and one significant drawback of the study was that the specimen types analysed were not carefully matched. A heterogeneous distribution of biofilm infections has been demonstrated, and it is likely that the specimens analysed did not have the same bacterial composition (Hall-Stoodley *et al.*, 2012; Thomsen *et al.*, 2010). The PRIS project made it possible to design a prospective study in a way making possible direct comparisons between different specimen types and between culture-based methods and 16S *rRNA* sequencing (Larsen *et al.*, Draft)[Paper 3].

Box 1 Parameters to be considered for the evaluation of a diagnostic test (Peeling et al., 2010).

Evaluation of a diagnostic test
Evaluation of a diagnostic test is dependent on the following parameters to give a proper picture of the actual performance for the intended purpose.

- *Test performance*
  Sensitivity, specificity, positive and negative predictive value
  The test has to cover both symptomatic and asymptomatic patients. The specificity can be more or less important depending on low cost and side effect of the treatment.

- *Usability*
  The usabillity of the diagnostic test should be evaluated in context of the clinical setting, it is attented for. Including parameters as time consumptions, human vs. machine, storage, and shelf lifetime.

- *Study group for evaluation*
  The study group should resemble the population in which the test will be applied. The group should be clearly defined with inclusion and exclusion criteria and should have a proper size.

- *The reference standard* should be clearly defined.

2.2.1.1 Sonication of joint prosthesis components

The increased attention to biofilm formation on foreign bodies has brought prosthesis components in the foreground for diagnostic testing (Høiby *et al.*, 2015;

Osmon *et al.*, 2013). Sonication and quantitative culture of sonication buffer have been applied to joint prostheses, spine implants, and pacemakers (Piper *et al.*, 2009; Rohacek *et al.*, 2015; Sampedro *et al.*, 2010; Trampuz *et al.*, 2007; Tunney *et al.*, 1998).

The laboratory handling requires suitable sterile containers for transport, sterile buffered saline to submerge the component (different variations have been published (Larsen *et al.*, 2012)[Paper 1]), vigorous vortexing, sonication (40 kHz) and a second turn of vortexing (Figure 3). Aliquots of the sonication buffer are subsequently cultured on a range of media in a quantitative manner. Alternatively, blood culture bottles have been used for incubation (Portillo *et al.*, 2015).

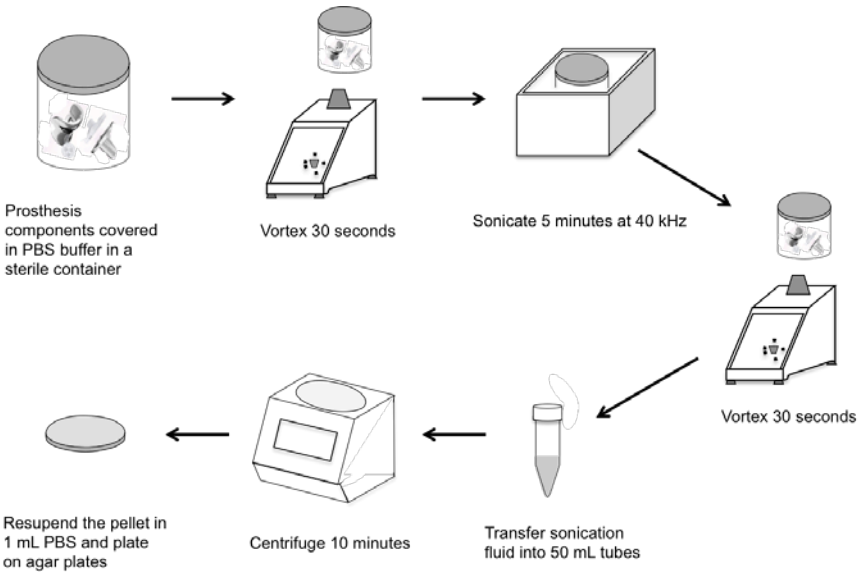


Figure 3 Sonication procedure used for knee and hip prostheses at the Department of Clinical Microbiology, Aalborg University Hospital in the PRIS project (Xu, 2014)

Sonication of prosthesis components has been shown to increase the sensitivity of culture compared to tissue biopsies, probably due to 1) the mechanical

dislodgement of bacteria, and 2) the enlarged sampling area from the entire surface of the prosthesis component following by 3) the concentration of bacteria by centrifugation of sonication buffer (Table 4) (Portillo *et al.*, 2013; Tunney *et al.*, 1998; Zimmerli *et al.*, 2004). Sonication (Figure 3) may accelerate growth resulting in faster positive culture reports compared with other specimen types from the same infection (Gomez & Patel, 2011). The explanation may be disruption of the cell wall/membrane, which initiates the transcription system for repair and thereby restarts persister cells in the biofilm and accelerates the growth rate of bacteria in the biofilm (Pitt & Ross, 2003).

Sonication has underwent some modifications over time, most significantly by addition of vortexing before and after sonication (Portillo *et al.*, 2013). Monsen and co-workers (Monsen *et al.*, 2009) showed a sonication period of 5 minutes to be a compromise covering both Gram positive and Gram negative bacteria, but the technique could be even more sensitive for the culture of Gram positive bacteria by increasing the sonication time (e.g. *S. aureus* may tolerate up to 20 min of sonication).

As mentioned before, the sonication buffer from prosthetic components is not only suited for culture by use of solid and liquid media, but also for inoculation of blood culture bottles similar to cultures of joint fluid (Font-Vizcarra *et al.*, 2010; Hughes *et al.*, 2001; Peel *et al.*, 2016). Inoculation of sonication fluid into blood culture bottles has been shown to be more sensitive than culture using standard media (as well as culture of tissue biopsies) with a gain especially of fastidious bacteria and bacteria exposed to antibiotics (Levine & Evans, 2001; Portillo *et al.*, 2015). Sonication buffer has also been found applicable for molecular diagnostics ranging from PCR-based methods including multiplex PCR to broad range 16S *rRNA* next generation sequencing (NGS) with notably high sensitivities (70 to 95%) and specificity (of 98-100%), which is generally a better performance than seen for culture-based studies using tissue biopsies and sonication of prosthesis

components (Achermann *et al.*, 2010; Cazanave *et al.*, 2013; Gomez *et al.*, 2012; Tunney *et al.*, 1999).

2.2.1.2 Prolonged culture incubation time

The choice of incubation time is a compromise between different concerns: Extended incubation increases the chance of growth of slow-growing or recuberating microorganisms, but may also increase the risk of contamination. For laboratory managers additional concerns include capacity of aerobic and anaerobic incubation facilities and workload due to multiple inspections, but a more important concern is the delay of a final diagnosis and treatment plan for the patient. As long as infection is not ruled out, the patient may be hospitalised for intravenous antibiotic treatment. Nevertheless, the attention to biofilm bacteria has led to gradual extension of standard incubation time (Hall-Stoodley *et al.*, 2012; Larsen *et al.*, 2012)[Paper 1]. Butler-Wu and co-workers showed an app. 30% increase in cultures positive for *P. acnes* when the incubation period for specimens from shoulder prosthesis infections was extended to 14 days instead of 7 days (standard); this observation is within range of other studies (Schäfer *et al.*, 2008; Skovby *et al.*, 2011). Not only anaerobic bacteria may need prolonged incubation time: In one patient in our own study (Larsen *et al.*, Draft)[Paper 3] *S. epidermidis* was obtained by culture on day 10 from the sonication fluid from the prosthetic component and first on day 14 from tissue biopsies. The cultures were clearly in a biofilm mode of growth as shown by several types of colony morphology, which may explain why the patient had vague symptoms from a knee arthroplasty for more than a decade (Larsen *et al.*, 2013)[Paper 2].

2.2.1.3 Results from the PRIS study

In the PRIS study (Larsen *et al.*, Draft)[Paper 3] five different specimen types were investigated, joint fluid, soft-tissue biopsies, prosthetic swab (*in situ*), the prosthetic component, and bone biopsies. All specimen types were cultured for a

period of 14 days and were investigated for the presence of bacteria by 16S *rRNA* broad range PCR followed by sequencing if positive.

The optimal specimen set for culturing was found to be joint fluid, soft-tissue biopsies, and the sonication fluid from the prosthesis component. Of note, neither the prosthetic swab nor bone biopsies contributed to the diagnosis (Figure 4). We found no single specimen type to be positive in all PJI patients, conversely, the different specimen types contributed differentially to the diagnosis of PJI depending on the microorganism. The time to culture positivity depended both on the specimen type and the microorganism. For early and acute onset infections with e.g. *S. aureus. Streptococcus* spp. and *Enterococcus faecalis* cultures of all specimens might be positive on day 1 or the microscopy of the joint fluid might indicate the infecting microorganism. The benefit of a broad collection of specimen types was largest in cases with slow infection (e.g. *S. epidermidis* and *P. acnes*) as well as in atypical and chronic cases. Dividing patients based on time of onset (see page 19) could be a rational sampling strategy prioritizing resources for patients in the grey area. The prolonged incubation period only added three extra patients (3/42, 7%) because of the contribution from different specimen types (Figure 5)

The comparison between culturing and 16S *rRNA* sequencing showed that the latter only added a few extra findings to the culture reports. This method can therefore be restricted to cases where this contribution is essential, e.g. chronic cases especially with a protracted history and cases who have been exposed to antibiotics before sampling.

In general, contributions from 16S *rRNA* sequencing have been restricted to liquid specimens which might be due to a much higher ratio between human and bacterial DNA in the final DNA extract from tissue biopsies compared to joint fluid and sonication fluid obtained with prosthetic components (Bémer *et al.*, 2014; Ryu *et al.*, 2014).

In the near future the focus must remain be on the difficult PJI cases where diagnosis remains a challenge.

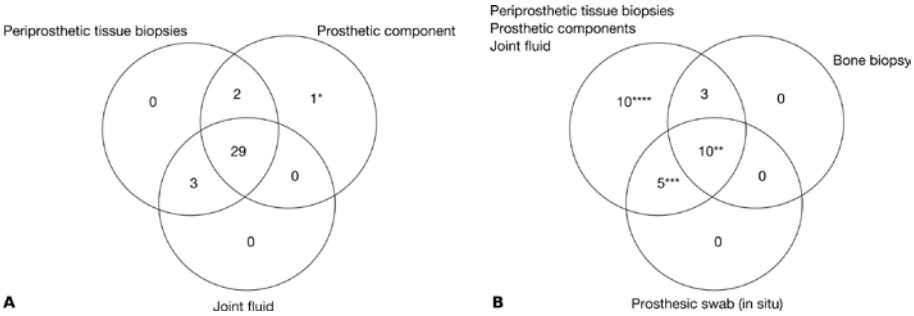


Figure 4 Contribution to positive results in cases where all specimens are present. (A) 35 cases with complete sets of soft tissue biopsies, prosthetic component, and joint fluid. (B) In 28 cases all specimen types were collected; neither bone biopsies nor prosthetic swabs contributed additional information (Larsen *et al.*, Draft)[Paper 3].

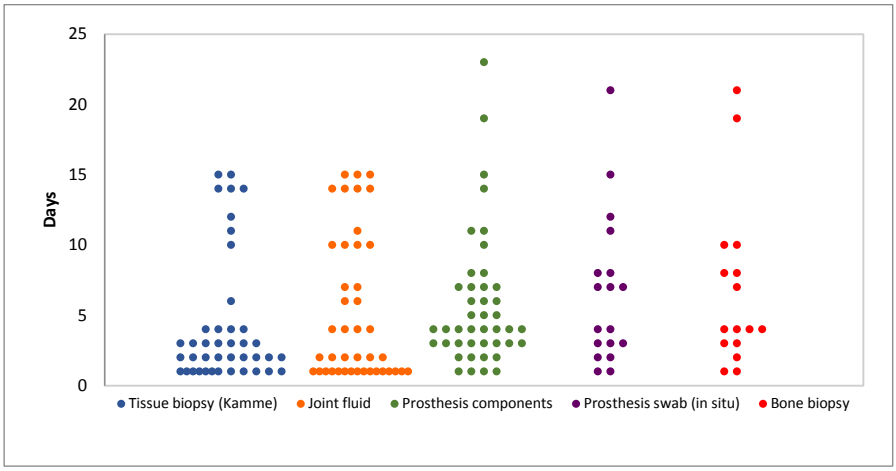
2.2.2. PCR based diagnostics for PJI

Molecular techniques for the diagnosis of PJI have not yet become standard in most laboratories (Bémer *et al.*, 2014) despite the fact that PCR tests have been applied since the late 1990s (Tunney *et al.*, 1999) (Table 4). The reported sensitivities show a wide variation (range 16-100%) depending on specimen type and the PCR technique used. Additional factors that may be conducive to the variation are selection of patients, experimental design, comparability of specimens, and the assays' technical parameters. Taking these limitations in consideration, molecular methods are still likely to be valuable supplements to conventional clinical microbiological diagnostic.

Generally, two different strategies have been applied in clinical microbiology (Table 4). The most frequent is based on multiplex qPCRs targeting common causative microorganisms by a combination of broad range 16S *rRNA* gene assays and species specific assays for *S. aureus*, CoNS, *Streptococcus* spp., anaerobic bacteria such as *P. acnes*, and 18S *rRNA* gene for fungi. The alternative strategy is based on

broad range 16S *rRNA* gene PCR screening followed by sequencing of the positive specimens.

A



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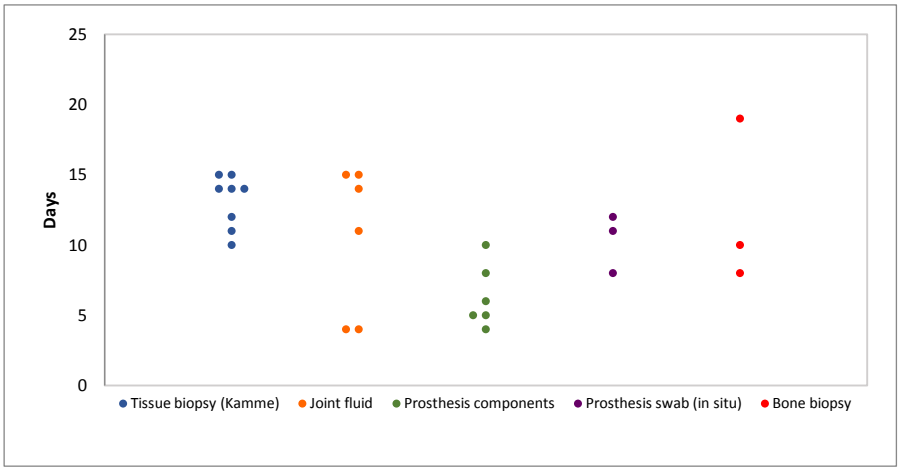


Figure 5: Distribution of positive specimens in relation to the day of culture positivity. (A) All positive specimens for all culture positive cases. (B) 8 culture positive cases who were not positive by our gold standard of periprosthetic tissue biopsies at day 6 (Larsen *et al.*, Draft)[Paper 3].

2.2.2.1 Multiplex PCR

Multiplex PCR has been used successfully for PJI in several studies and especially for patients receiving antibiotic treatments prior to surgery (Achermann *et al.*, 2010; Cazanave *et al.*, 2013; Portillo *et al.*, 2012; Ryu *et al.*, 2014). In particular, Cazanave et al. found it advantageous to combine broad range primers and specific sets of primer pairs for the most common PJI bacteria.

The choice of species to be targeted by multiplex PCR assays is dependent on knowledge of the spectrum of bacteria previously linked to PJI, and rare bacteria will not be detected by this diagnostic approach. However, the method is expedient and has the potential for providing same-day diagnosis. In this sense multiplex PCR diagnostics is superior to bacteriological culture.

2.2.2.2 Broad-range 16S *rRNA* PCR followed by sequencing

Historically, broad-range 16S *rRNA* gene PCR has been popular for detection of bacteria since the original work of Tunney et al. (Tunney *et al.*, 1999). Detection of the 16S *rRNA* gene is a distinct marker of bacterial presence, and subsequent sequencing allows phylogenetic classification of bacteria based on the heterogeneous regions (Harris & Hartley, 2003). Previously, the step after amplification has either been cloning of 16S *rRNA* followed by Sanger sequencing (Dempsey *et al.*, 2007; Fenollar *et al.*, 2006; Vandercam *et al.*, 2008) or direct Sanger sequencing with and without RipSeq analysis if more that two bacterial species was present (Fenollar *et al.*, 2006; Gomez *et al.*, 2012).

The use of more advanced sequencing methods will be descripted later.

2.2.2.3 Drawbacks of PCR based diagnostics

In the literature it is often emphasised that the primary benefit of using PCR-based diagnostics is a faster diagnosis of the infecting microorganism(s) compared to culture methods (Hall-Stoodley *et al.*, 2012) . However, there are other important aspects of molecular methods that need to be studied.

Ryu et al. has briefly mentioned the ratio of human DNA to bacterial DNA being a problem when working with tissue specimens (Ryu *et al.*, 2014). In specimens with few bacteria the amount of bacterial DNA can be very low in the final DNA extract either due to insufficient DNA extraction or 'drowning' in the overload of human DNA leading to false negative results. Using different DNA extraction kits we found both the detection limit and the background contamination profile (se next section) to vary, too. Specifically, we compared the performance of MolYsis complete kit 5 (Molzym, Germany) with the Dneasy Blood & Tissue kit (Qiagen, USA) for DNA extraction of complex human specimens, and with the first kit we obtained PCR bands of correct size after broad range PCR while there were many unspecific bands from the extractions with the latter one (Xu *et al.*, 2016a).

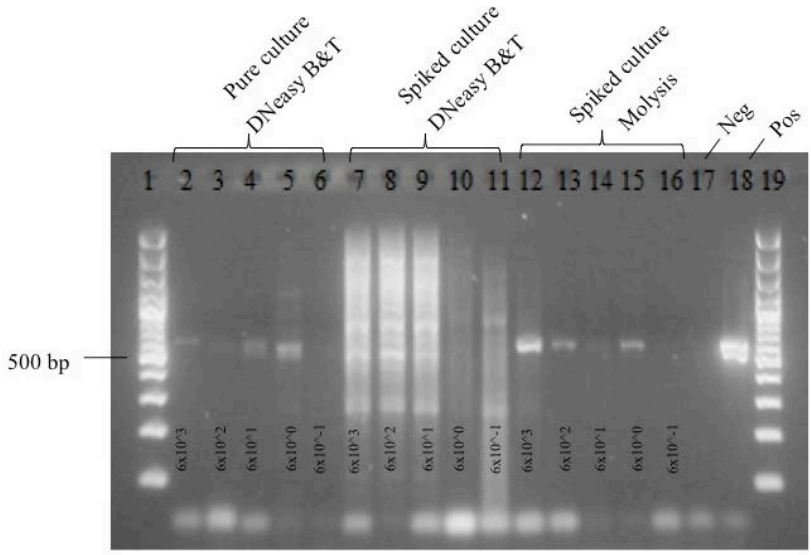


Figure 6**.** Agarose gel electrophoresis of the products from a broad range 16S rRNA gene PCR amplified using 26F and 518R primers and 30 cycles. DNA was extracted from pure cultures of *Enterococcus phoeniculicola* and complex human specimens. With two different DNA extraction kits; Dneasy Blood & Tissue kit (Qiagen, USA) and MolYsis complete kit 5 (Molzym, Germany). Strong smears from spiked culture extracted with Dneasy Blood & Tissue kit indicate clear influence of human DNA on PCR (Xu *et al.*, 2016a)

Table 5 Advantages and disadvantages of the culture-independent methods covered in this thesis (modified after Rudkjøbing, 2012).

	Advantages	**Disadvantages**
FISH	Fast (< 1 day) Cost efficient Probes can be designed for each taxonomic level Less sensitive to contamination than PCR-based methods Provide information about cell morphology, number, spatial distribution or the cellular environment	Insufficient probe penetration and hybridization may result in low signal intensity Low physiological state may result in low rRNA content and hence low signal intensity. High detection limit. Often difficult to detect low numbers of bacteria. Less sensitive than amplification based methods Potential autofluorescence of biomaterial or host tissues
QPCR	Fast (< 1 day) No post-amplification handling High sensitivity Can provide quantitative results Many available assays Can be multiplexed	Only targeted species will be detected Sensitive to contamination Sensitive to PCR inhibitors
PCR/ESI-TOF-MS (Ibis)	Fast (< 1 day) Can potentially identify all species by combination of broad and narrow range primers Can provide quantitative information Automated	Not widely tested Costly Data analysis is a black box
Broad-range 16SrRNA PCR based methods		
Direct Sanger sequencing (followed by RipSeq)	Fast (< 1 day) High sensitivity Long read length Can provide species information by BLAST sequences in databases	Generally requires single target sequence in one sequencing reaction. In the case of polymicrobial infection, RipSeq can be used to resolve mixed chromatogram (up to 3 species) Costly
Cloning and Sanger sequencing	Can separate individual PCR fragments Near full-length 16S rRNA gene sequence can be obtained Can provide species information by BLAST sequences in databases	Costly Time consuming and labour intensive (> 3 days) An adequate number of clones need to be sequenced to avoid missing low abundant species
Next generation sequencing	High throughput High species resolution Can provide species information by searching databases Continuous fast improvement of the technologies	Time consuming (>2 days) Require extensive bioinformatics analysis High cost per run Read length is limited to around 500 bp currently

2.2.2.4 A bioinformatics approach for removal of contamination and estimation of cut-off, an example

16S *rRNA* amplicon sequencing is used to identify and compare bacteria present in the specimen. The microbial diversity can be compared between different specimens independent of culturing and nonculterable bacteria can be

investigated. The 16S *rRNA* genes are amplified by PCR using broad-range primers (v1-v3 region, 30 cycles) including barcodes to identify the specific specimen and sequencing can be applied. During extraction of DNA the specimen can be exposed to contamination both from the DNA extraction kit itself and from the laboratory environment even though all precaution have been taken. In NGS, the background contamination profile has been shown to have its individual bacterial fingerprint for every laboratory and every molecular kit combination in play, especially for specimens with a very low amount of target bacteria (Salter *et al.*, 2014; Xu *et al.*, 2016a). The above problems are not relevant for specimens containing high amounts of bacteria because the target will outcompete less abundant contaminants in the specimens in the initial PCR preparation. However, for the specimens with low amounts of bacterial DNA, the contamination can become significant in the dataset, and a trained bioinformatician is needed to safeguard the interpretation of the results.

For the correct classification of true amplification-positive and amplification-negative specimens in the PRIS study the contamination profile was ascertained in order to distinguish the amount of background reads from the true positive specimens (Larsen *et al.*, Draft)[Paper 3].

For this purpose we analysed a subset of specimens investigated by molecular methods in the PRIS dataset (32 patient specimens of all specimens types, 16 negative DNA extraction controls, one negative PCR control, and one positive DNA extraction control). For every specimen, the number of reads sequenced was normalised to the amount of DNA (in nanograms) pooled for sequencing, and net the numbers were plotted against the DNA input. Figure 7 shows the results from a data analysis using 97% similarity for classification of OTU (data from all sequences top left and specimens with less than 500 reads/ng DNA input top right). There was an overlap between the amount of reads from the negative controls and some of the specimens. After removing the OTUs with less than 100 or 200 reads a clear separation was seen between the true positive (above 200 reads/ng DNA)

specimens and negative controls together with negative specimens, and it is possible to set a cut-off of 200 reads/ng DNA input for these runs, to distinguish the amplification-positive specimens and the amplification-negative specimens. For the specimens just above 200 reads/ng DNA input and less than 500 reads there is a grey area and individual estimation has to be made, taking into account other specimens from the same patient, the microorganism in question, and the results obtained by culture as well as clinical evaluations. Similar results were achieved for 99% similarity of OTU (not shown).

The above estimation can be applied to all specimens in the dataset regardless of the sequencing results, but looking specific at the contamination fingerprint in the negative controls and thereby at the OUT collection which have to be removed from the final data, the contamination profile shows typical environmental bacteria evenly distributed across the negative controls and the specimens (with low amount of reads). Also more common bacteria such as *Staphylococcus* sp. and *Propionibacterium* sp. (possible *P. acnes*) are represented with reads in every negative control. This gives an indication that a specific cut-off has to be set for these two bacteria.

Specimens positive for *Staphylococcus* sp. are often easy to recognise. The numbers of reads tend to be above 1000 reads/ng DNA input (Figure 8, top left) and this is characteristic for true positive specimens with all types of bacteria. For the specimens below 150 reads/ng DNA input, the cut-off was 80 reads/ng DNA input independent of the removal of OTUs with less than 100 or 200 reads for *Staphylococcus* sp. OTU. The grey area between 80-150 reads/ng DNA input needs an individual estimation in relation to the overall diagnostic results.

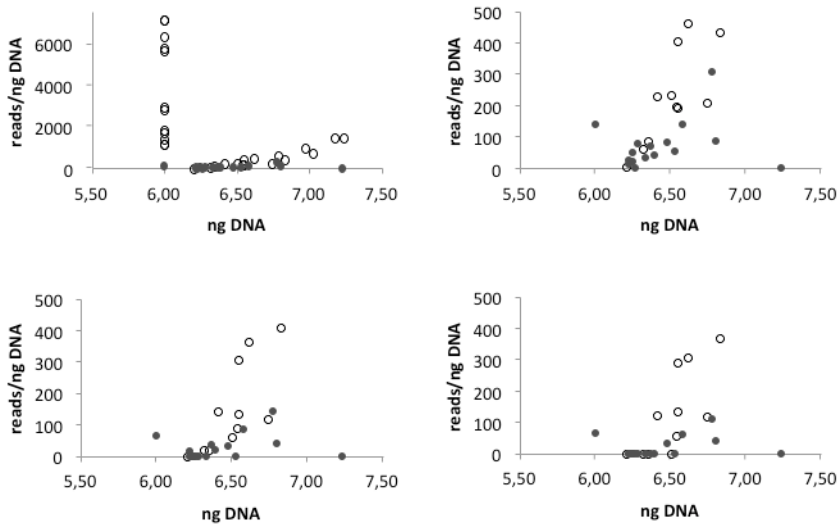


Figure 7. Estimating the cut-off for all sequences. Reads are normalised against ng DNA input of the PCR product into the multiplex for sequencing and plotted against the amount of ng DNA input. Open: specimens, closed: negative controls. Top left: total reads, all specimens. Top right: total reads, section of reads below 500 reads/ng DNA. Bottom left: OTUs less than 100 reads removed. Bottom right: OTUs less than 200 reads removed.

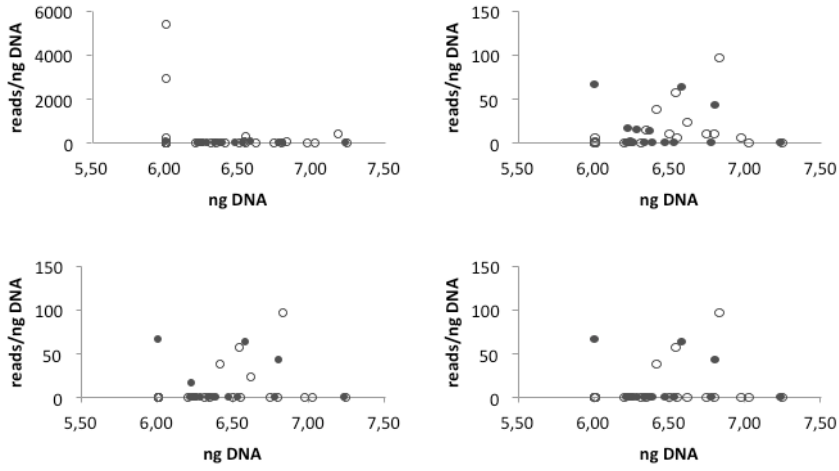


Figure 8. Estimating cut-off for *Staphylococcus* sp. Reads achieved from sequencing are normalised against ng DNA input of the PCR product into the multiplex for sequencing, and

plotted against the amount of ng DNA input. Open: specimens, closed: negative controls. Top left: total reads, all specimens. Top right: total reads, section of reads below 150 reads/ng DNA. Bottom left: OTUs less than 100 reads removed. Bottom right: OTUs less than 200 reads removed

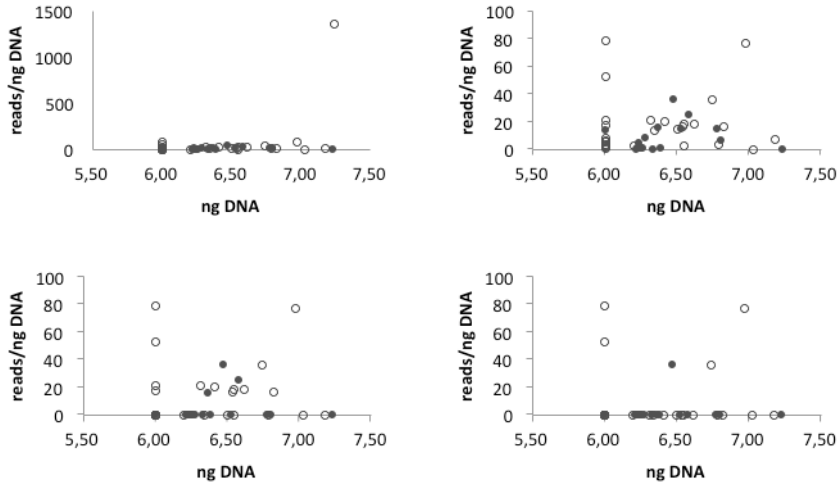


Figure 9. Estimating cut-off for *Propionibacterium* sp. Reads achieved from sequencing are normalised against ng DNA input of the PCR product into the multiplex for sequencing, plotted against the amount of ng DNA input. Open: specimens, closed: negative controls. Top left: total reads, all specimens. Top right: total reads, section of reads below 100 reads/ng DNA. Bottom left: OTUs less than 100 reads removed. Bottom right: OTUs less than 200 reads removed

For the *Propionibacterium* sp. (Figure 9), two specimens in the data set were convincingly positive (top left; only one within the plotted area). The rest of the specimens had 100 reads/ng DNA input or below. They were plotted as above, and when all *Propionibacterium* sp. OTUs with less than 200 reads were removed, it was possible to see a separation between the true negative specimens and specimens in the grey area. A cut-off of 60 reads/ng DNA input was now feasible with a grey area between 60-100 reads/ng DNA input where specimens need further evaluation.

This kind of evaluation has to be made for every sequence dataset, especially in the light of the results mentioned above by Salter *et al.* (2014). Optimally, the contamination profile should be evaluated every run and more pragmatically, at least every time changes are made in the preparation of specimens for sequencing.

2.3. Explorative approaches

Investigations of non-culture based methods for the diagnostic of PJIs have been on-going for the last 20 years. In the pursuit of an easy and less invasive diagnostic method, different biomarkers such as C-reactive protein (CRP), joint fluid leukocyte count and the more explorative α-defensin 1-3 have been investigated. However, this thesis will not address the biomarkers, but focus on the diagnostic microbiology in PJI in hips and knees (Cipriano *et al.*, 2012; Deirmengian *et al.*, 2014; Parvizi *et al.*, 2011a, b).

2.3.1. PCR/ESI-TOF-MS

PCR/ESI-TOF-MS also called IRIDICA (Abbott) combines PCR with high-performance electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). Using Bac spectrum kit, unknown DNA is amplified with several primers covering both broad range and also more species-specific sequences including *Candida* spp. and selected antibiotic resistance genes (e.g. *mecA* and *vanA*) (Ecker *et al.*, 2008). After PCR, the PCR products are automatically desalted. Then the ESI denaturates each product, which is subsequently weighed by TOF-MS with a precision enough to calculate the compositions of nucleotides. Based on a triangulating algorithm bacterial species are identified as a result of the calculated base compositions of the multiple PCR products and a database search. By comparing MS peaks in relation to internal standards, it is possible also to quantify bacteria in the specimens (Ecker *et al.*, 2008).

Table 6 Overview of the applied methods and results for a PJI case study (Xu et al., 2013) . The numbers in the brackets refer to the number of analysed clones belonging to the species.

Procedure	Method	Specimen	Organism	Comments
Debridement with prosthesis retention (housecleaning)	Culture	5 Tissue biopsies	-	Culture was negative most probably due to the ongoing antibiotic therapy.
	Cloning	Joint fluid	S. dysgalactiae subsp. equisimilis (72)	A total of 72 clones were sequenced and analyzed. All of them identified the same species.
		Prosthesis swab	S. dysgalactiae subsp. equisimilis (69); Propionibacterium granulosum (7); Kocuria palustris (5); Paracoccus sp.(3); P. acnes (3); Anaerococcus sp. (2); Achromobacter xylosoxidans(1); Peptoniphilus sp. (1); Uncultured bacterium (2)	A total of 83 clones were sequenced and analyzed.
	Ibis	Tissue biopsy	Streptococcus pyogenes	
		Bone biopsy	S. pyogenes	
	Pyrosequencing	Tissue biopsy	S. dysgalactiae	
		Bone biopsy	S. dysgalactiae	

The system is predicted to fulfil the requirements for molecular diagnostic in clinical microbiology. Xu *et al* 2013 (Table 6) applied the research system Ibis Biosciences T5000 biosensor (now IRIDICA, Abbott) on a PJI (hip prosthesis) and made a comparison with culture, cloning, and pyrosequencing. Cultures from peroperative tissue biopsies were negative probably because of on-going antibiotic therapy at the time of surgery. Cloning from the prosthetic component was polymicrobial with the main products derived from *Streptococcus dysgalactiae*. The majority of the pyrosequencing reads were also *S. dysgalactiae*. The Ibis identified *Streptococcus pyogenes*, as the most likely species in tissue and bone biopsies, which indeed was close taxonomically to the causative agent *S. dysgalactiae*.
In the newer versions of the Ibis the specificity remains a challenge, and the method is still prone to contamination during handling which requires a cautious

interpretation of the results (Brinkman *et al.*, 2013; Greenwood-Quaintance *et al.*, 2014; Jacovides *et al.*, 2012).

2.3.2. Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) is a powerful tool to investigate biofilm. It is well suited for the study of the architecture of biofilm formed in the human body in contact with tissues as well as humoral and cellular immune mediators (Hall-Stoodley *et al.*, 2012; Moter & Göbel, 2000). The method was not a part of the clinical routine but was applied as an explorative method in the PRIS project. With the FISH method we are able to detect specific nucleotide sequences by fluorescence labelled probes. An often used target site is ribosomal RNA primarily because of the high numbers of ribosomes in bacteria, but also because of the possibility of targeting more or less conserved regions (Woese, 1987)
FISH is less sensitive to contamination compared with PCR based techniques and has therefore been used for demonstration of biofilm directly on implants such as prostheses or central venous catheters (Stoodley *et al.*, 2011; Thomsen *et al.*, 2011). The limited area of a medical implant, which can be investigated by microscopy, calls for preparatory techniques such as scraping the surface or sonication combined with centrifugation.
Peptide Nucleic Acid-probes (PNA-probes) are mostly used for direct identification of bacteria growing in blood culture bottles for accelerating the time for diagnosis (Deck *et al.*, 2012). Diagnostic kits are available (AdvanDx, USA), but the commercially available probes are still limited. The FISH method demonstrates the aggregation of cells (Figure 10), and in an optimal combination with confocal microscopy the biofilm composition and distribution can be visualised (Thomsen *et al.*, 2011). To overcome heterogeneous distribution of bacteria on the surface of infected prostheses, we used a modified PNA-FISH protocol. With this protocol we have been able to lower the detection limit to $\geq 10^2$ CFU/mL via concentration of

the sonicate specimen on a polycarbonate filter, (Figure 10 bottom) (Larsen *et al.*, 2014b).

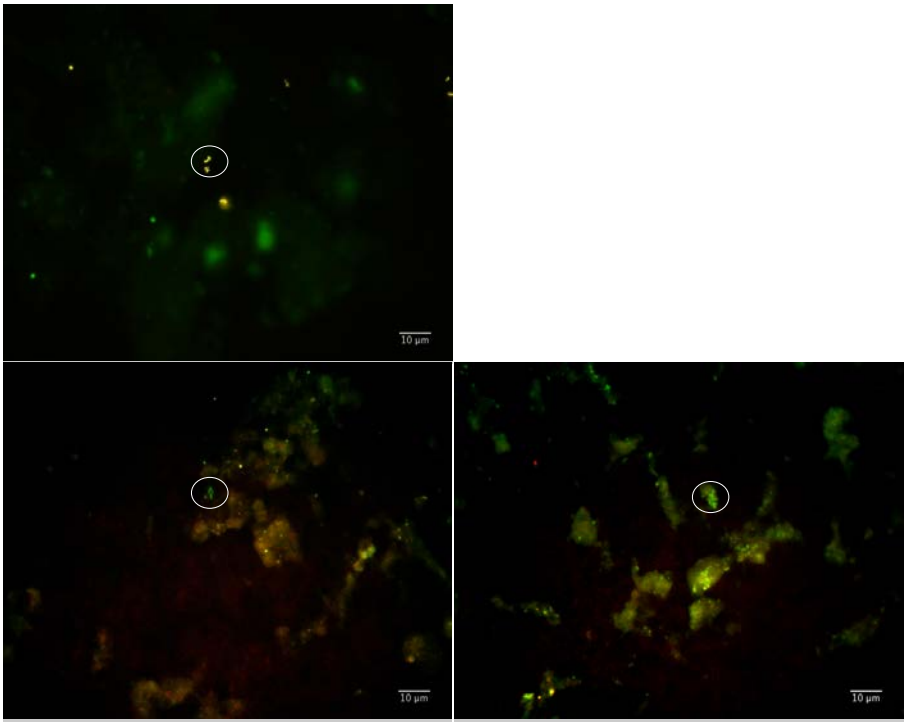


Figure 10. Demonstration of single cells and microcolonies by use of different PNA-FISH probes. (Top): *Enterococcus faecalis* PNA-FISH probe (red) and universal bacterial PNA-FISH probe (green). (Bottom both pictures): *Staphylococcus aureus* PNA-FISH probe (green) and universal bacterial PNA-FISH probe (red). *S. aureus* should appear yellow when both probes hybridised. However, only few cells are yellow which can be caused of poor hybridization or shift in focus layer during documentations. No red cells indicate that it is a S. aureus monoinfection (Larsen et al., 2014b).

2.4. Knowing the 'enemy'

The literature summarised in Table 4 indicates that culture methods in combination with molecular methods and a range of specimen types can provide a more comprehensive picture of microorganisms involved in PJI. This augments the understanding of the pathogenesis of biofilm infections. In an allied field of

microbiology, mapping metabolic pathways in the genome of unculturable anaerobic bacteria has led to development of media overcoming restrictions of growth (Sommer, 2015). Other studies have suggested that the presence of some bacteria does not necessarily implicate an infection that requires treatment (Burmølle *et al.*, 2010; Røder *et al.*, 2015). Others believe that some microorganisms might act synergistically in polymicrobial infections, and the bacterial diversity itself may promote a chronic infection in addition to the pathogenicity of the individual bacteria (Brogden *et al.*, 2005; Burmølle *et al.*, 2014; Ehrlich *et al.*, 2005). Since the contribution of individual bacteria in the pathogenesis of polymicrobial infection has not yet been explored extensively, different "omics" strategies might help to show the hidden life of the enemy and possibly direct new treatment strategies for biofilm-related infections.

2.4.1. Towards the optimal diagnostic strategy

All diagnostic methods have advantages and limitations. Few molecular techniques are used regularly or even routinely for the diagnostic of PJI, and thus most diagnoses are still based on culture. The conventional techniques in clinical microbiology are based on isolation of the most prevalent planktonic and fast growing pathogens on/in agar or fluid media, which might miss more fastidious and/or slow growing bacteria together with more uncommon non-culturable bacteria. For the diagnosis of PJI, future diagnostics have to overcome these limitations.

The optimal diagnostic tool should be expedient, equally applicable to common specimen types, both sensitive and specific, and robust to variations in the clinical setting as well as in the diagnostic laboratory. It should provide the species of the infecting organism(s), phenotypic form (biofilm/planktonic), pathogenic factors expressed, and potential resistance mechanisms, thus providing the best treatment

options. NGS and PCR/ESI-TOF-MS might fulfil some of these requirements, at least in theory.

NGS techniques have via whole genome sequencing (WGS) been used for the investigation of host-pathogen interactions and the epidemiology of outbreaks, but the use for routine clinical diagnostics is still in the making (Punina *et al.*, 2015). Over the years the price of sequencing has been dropping (National Human Genome Research Institute, USA), and at present the bottleneck for using sequencing techniques is the analysis and interpretation of the large data output, together with the estimations of the clinical impact.

In the foreseeable future, culture techniques will remain a mainstay for the diagnosis of PJI, as they are affordable and cover most of the causative agents. Molecular methods can be reserved for a selected group of patients in whom culturing has failed or is likely to fail. The most pertinent question is therefore which combinations of specimen types and techniques are most effective (being daring enough to refer to this as the optimal combination).

In our study (Larsen *et al.*, Draft)[Paper 3] the optimal specimen set for culturing was a combination of joint fluid, soft tissue biopsies and the sonication fluid from the prosthesis component. Others studies (Bémer et al., 2014, 2015; Butler-Wu et al., 2011; Font-Vizcarra et al., 2010; Gallo et al., 2008; Gomez et al., 2012; Hughes et al., 2001; Peel et al., 2016; Roux et al., 2011; Schäfer et al., 2008; Trampuz et al., 2007) have investigated the use of individual specimen types against each other but no one has addressed the question of the optimal specimen set taking in account that no technique and/or specimen cover the entire spectrum of PJI infections. In our own study (PRIS) we investigated several parameters, and the sampling in parallel of different specimen types allowed direct comparison between specimen types and methods (foremost culture media, an extended incubation period. and 16S *rRNA* sequencing) Out of the 43 PJI cases in the study, 12% (5/43) had culture-positive project specimens within the standard incubation

period of 6 days. Concurrently, cultures of standard soft tissue biopsies confirmed 67% (29/43). Three additional cases had late culture-positive project specimens (i.e. after 7 to 14 days of incubation). Thus, in all 19% (8/43) had culture-positive project specimens; this figure is comparable to other studies (for further details, see summary of Paper 3 on page 62). (Butler-Wu *et al.*, 2011; Schäfer *et al.*, 2008). It is potentially an important observation that a broad collection of specimens may shorten time to positivity and hence favour shorter incubations times. The comparison between culturing and 16S *rRNA* sequencing showed that sequencing only added only a few extra findings to the culturing reports. Compared to culturing, the molecular methods are relatively costly and time consuming and require many working hours of skilled laboratory personal. Therefore the use of this method is suggested to be restricted for cases in whom the results can be crucial, e.g. long-term chronic cases and cases exposed to antibiotics before sampling.

Chapter 3. Understanding microbial biofilms

In chapter 2 the focus was on detection of bacteria present in biofilms, here the focus will be on revealing how they survive and grow *in vivo*. One of the key factors for survival in a biofilm infection is the ability to utilise proper carbon sources and electron acceptors from the environment within the human host and to maintain a proper environment for survival. Exploring the metabolism of bacteria *in vivo* may help to understand their 'secret life' and how to fight them. Several different approaches can help to elucidate parts of this question. By understanding the metabolic activity in addition to the virulence properties, new targets for bacterial eradication may become available, offering alternatives to antibiotics, which often fail biofilm infections (Rohmer *et al.*, 2011).

3.1. Investigations of biofilm infections

A variety of investigation models contribute together with studies in patients to an increasing body of knowledge regarding implant-related infections within different areas. This multifaceted approach is needed to cover the complexities of biofilms *in vivo. In vitro* models are simple to set-up and suited for investigation of biofilms under standardised and systematically varying conditions, e.g. bacterial-biomaterial-mammalian cell interactions step by step in short-term perspective, whereas the *in vivo* models allow the study of long-term interactions and biological integrations (Lebeaux *et al.*, 2013; Roberts *et al.*, 2015; Subbiahdoss *et al.*, 2013).

3.1.1. *In vitro* models

The advantages of *in vitro* models are low-cost and simplicity of set-up with good possibilities for controlling single parameters within the system and maintaining a high throughput for screening purposes. A comprehensive comparison of different model systems for studying biofilm infection *in vitro* can be found in Roberts and co-workers (Roberts *et al.*, 2015). The disadvantages of *in vitro* models are

primarily the simplicity of the systems that does not resemble the complexity of *in vivo* situations, e.g. they do rarely reflect oxygen and nutrient gradients found in a 'real' biofilm infection (Roberts *et al.*, 2015). Another notable example is the fact that typical mushroom-shaped biofilm forms are seen in flow cells but never observed *in vivo* (Figure 11).

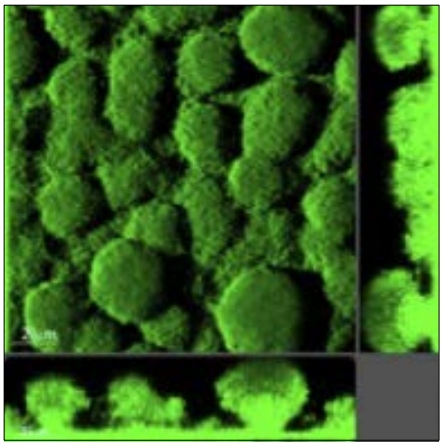


Figure 11 Confocal laser scanning micrographs of a 3-day old mushroom shaped biofilm formed by a *Pseudomonas aeruginosa* wild-type strain in a continuous flow cell system. The central image shows top-down view and the flanking images show vertical optical sections. The bar represents 20 µm (modified from Roberts et al. 2015, Bjarnholt, unpublished results)

For improving *in vitro* models to mimic the *in vivo* systems even more, a two-culture system offers the possibility of including both bacteria and mammalian cells. A relevant example is a model mimicking insertion of an implant with biofilm formation in the presence of mammalian cells (Subbiahdoss *et al.*, 2009, 2011). The authors found that the formation of biofilm by low-virulent *S. epidermidis* was dependent on the ratio between bacteria and mammalian cells, whereas highly virulent bacteria such as *S. aureus* and *P. aeruginosa* producing exotoxins often won the 'race for the surface' and were less dependent on the ratio between bacteria and mammalian cells.

The choice of model depends on the questions to be answered. However, results obtained with *in vitro* models should be extrapolated cautiously to *in vivo* infections, and in the end results must be confirmed *in vivo*. As an example the RNA gene expression profile from *in vitro* culture of *S. aureus* shows very distant relations to *in vivo* gene expression profiles of a human PJI and the implant infection in a guinea pig model. Even though *in vivo* expression was studied in so different mammalian hosts like guinea pig and man, *in vivo* gene expressions profiles clustered together (Larsen *et al.*, Draft)[Paper 4].

By use of *in vivo* animal models, the study of implant-related infections comes one step closer to human infections. The *in vivo* models are more complex systems with interplay between the host's immune defence and multiple physicochemical factors such as nutrient availability, oxygen tension, and pH. The unique combination of all these factors cannot be mimicked by *in vitro* systems, even with multi-culture approaches (Subbiahdoss *et al.*, 2013).

However, the accessibility of *in vivo* models is restricted by national and international legal regulations and special animal facilities are required. Consequently, such studies are time-consuming and require long-term planning from the experimental design through legal approval until the experiments are conducted and results are ready for analysis. In this process, the choice of model is most important. For the right choice, the biology of the experimental animal has to be considered together with animal behaviour as this might heavily influence the final results. A comprehensive comparison of different model systems for studying biofilm infection *in vivo* can be found in Lebeaux and co-workers (Lebeaux *et al.*, 2013). In our study (Larsen *et al.*, Draft)[Paper 4] the question asked was "How does the implant-related *S. aureus* biofilm infection behave when exposed both to the host's immune defence and moxifloxacin treatment?" To answer the question, an established *S. aureus* implant-related infection model was needed. Our choice was the tissue-cage model in guinea pigs (Zimmerli *et al.*, 1982), where the host response to the implant has already been described (Zimmerli *et al.*, 1984). This

facilitates interpretation of the results. It is well-known that differences in genetics and immune profiles between animal species give different results even with the same approach. Thus, establishing a long-term infection in a guinea pig model is more predictable compared to mouse and rat models in which spontaneous clearing of infection happens quite offend. The spontaneous clearance has not yet been observed in humans, and therefore the choice of the guinea pig model was deemed more relevant for the study of an implant-related infection (Zimmerli, 2014).

3.1.2. Investigating PJI *in vivo*

The study of biofilms *in vivo* is not reserved to animals, but is also feasible in humans in certain situations. However, one must be aware of the large diversity in patient background variables: age, gender, co-morbidities, various treatments, the presumed duration of the biofilm infection, and different infecting microorganisms. Such unique studies provide an insight into the "real world", but the generalizability remains questionable. Xu *et al.* (Xu *et al.*, 2016b) investigated a PJI in a patient by a combination of transcriptomics and joint fluid metabolomics. The study revealed a *S. aureus* infection thriving on amino acids in a low oxygen environment with surprisingly high levels of lactate and ethanol.

3.1.3. 'omics' methods

To study the metabolic activity within the infecting bacterial biofilm, precise tools are necessary. Within the recent years high throughput methods have become accessible for identifying processes and regulatory mechanisms that characterise the bacterial network in this type of infection. By combining genomics, transcriptomics, proteomics and metabolomics, we might be able to gain insight into the pathophysiology and virulence factors in biofilm infections (Figure 12).

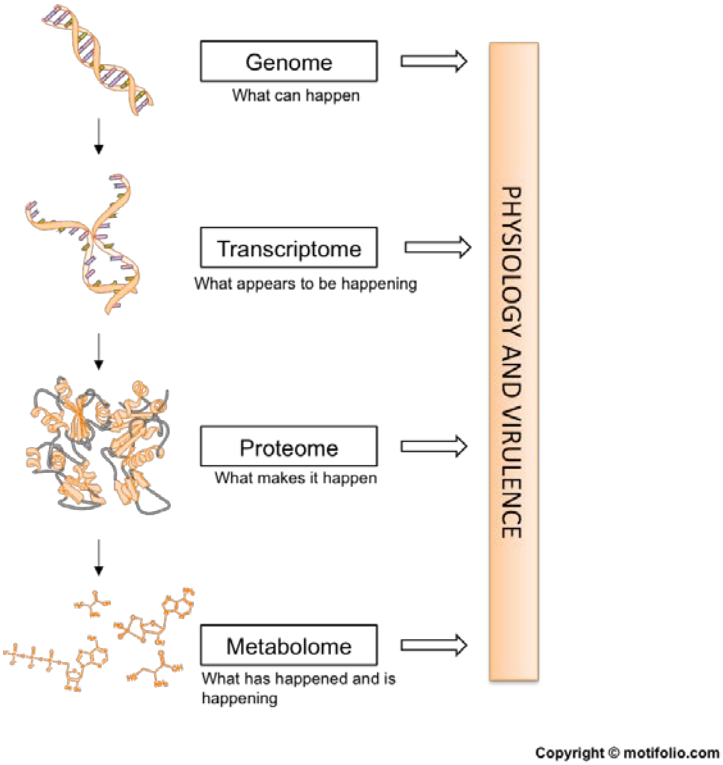


Figure 12 An integrated "omics" approach for understanding physiology and virulence of pathogens (Xu, 2014).

3.1.3.1 Genomics

Bacteriology has embraced NGS by using whole genome sequencing (WGS). Thousands of genomes are being sequenced and high numbers are already available for the more frequent bacterial species, allowing us to look into pathogenesis, ecology and evolution of the bacterial pathogens.

Recently Méric and co-workers (Méric *et al.*, 2015) analysed 324 genomes of *S. aureus* and *S. epidermidis* and showed that gene transfer between the two core genomes was rare and only nine such genes were found despite the fact that the two species have common niches on the human skin and upper airways. However, horizontal gene transfer was more frequent for mobile elements such as

pathogenic islands (such as the chromosomal cassette *mecA* encoding methicillin resistance).

The available genomes provide - via comparative genomics - the basis for further development of molecular diagnosis of specific pathogens and are also the backbone for studies including transcriptomics and proteomics.

3.1.3.2 Transcriptomics

The study of transcriptomics gives us insight into the bacterial response to the surrounding microenvironment by providing the gene expression profile of metabolic pathways as well as stress and virulence factors. It is a snapshot of the physiological state of the bacteria, which might reveal the new targets for fighting the infection (Waddell *et al.*, 2007).

Traditionally, DNA microarray has been used for the analysis of gene expression profiles of known genes in a fast manner. It has been used for many different bacterial species under different conditions *in vitro*. Isolating bacterial mRNA from *in vitro* cultures is more or less straightforward, but isolating the bacterial mRNA from host tissue in sufficient amounts for analysis is a big challenge (Gomez *et al.*, 2011). The use of DNA microarray is dependent of prior knowledge of the genome of interest based on the hybridization to oligonucleotides. High throughput RNA sequencing (RNA-Seq) is de novo sequencing which makes it feasible to generate millions of sequences independent of primers and thus allowing transcripts of both know and unknown sequences to be revealed (Bradford *et al.*, 2010; Croucher & Thomson, 2010). Still, the main drawback is the need for millions of cell as starting material, which may be difficult to obtain without access to pure cultures. Working with clinical specimens the ratio between the amounts of prokaryotic and eukaryotic RNA is critical. Only a few studies have managed to study the bacterial gene expression profile *in vivo* in animal infections (Jorth *et al.*, 2013; Szafranska *et al.*, 2014; Tong *et al.*, 2016; Yan *et al.*, 2013) and in human infections (Bisharat *et al.*, 2013; Lim *et al.*, 2013; Xu *et al.*, 2016b).

An example of transcriptomics is described in section 1.9.3.5. In general such studies provide a high-resolution genomic analysis of the bacterial pathogen under *in vivo* growth conditions but also provide insight into the metabolic pathways required for the bacteria to in survive in the course of infection.

3.1.3.3 Proteomics

By proteomics the goal is to describe protein expression quantitatively and if possible, to follow changes over time and during different conditions. The high throughput studies of proteins are basically based on two techniques, two-dimensional gel-based techniques, where proteins are separated by the isoelectric point and mass followed by mass spectrometry (MS). Non-gel based methods apply liquid chromatography and tandem mass spectrometry (LC-MS/MS) (Weston & Hood, 2004).

The bacterial proteome has been studied extensively *in vitro* under different conditions. Bénard and co-workers (Bénard *et al.*, 2009) studied differences in *S. aureus* gene expression profile between the planktonic and biofilm state, and they found an accumulation of surface-related proteins to be associated with biofilm. *In vivo* studies are more complicated due to the presence of host proteins, which is cumbersome to disentangle (Kolmeder & de Vos, 2014) even through several studies have managed to study the bacterial proteome in the intestinal tract in animals (Alpert *et al.*, 2009; Muth *et al.*, 2013; Roy *et al.*, 2008) and in human body fluids (Fouts *et al.*, 2012; Jagtap *et al.*, 2012; Xie *et al.*, 2008).

As an example, metaproteomic studies in the intestinal tract of mice revealed an ability of *E. coli* to upregulate utilization of several different carbon sources simultaneously with culture *in vitro* serving as a control (Muth *et al.*, 2013). The same pattern was also found in *Lactococcus lactis* in the gastrointestinal tract of mice (Roy *et al.*, 2008), and also showed a more diverse metabolism in the *in vivo* model.

3.1.3.4 Metabolomics

The metabolome is the sum of small molecules (<1500 Da) produced by an organism and can be seen as the most direct indication of a cell's metabolic state. In addition, novel pathways and quantification of flux within the metabolitic network can be explored by use of isotope-labelled metabolites (de Carvalho *et al.*, 2010). The intracellular metabolism can be studied with intact cells whereas the flux of metabolites to the surroundings can be determined from the supernatant. Techniques such as nuclear magnetic resonance (NMR) spectroscopy, capillary electrophoresis coupled to mass spectrometry (CR-MS), gas chromatography coupled to mass spectrometry (GC-MS), and liquid chromatography coupled to mass spectrometry (LC-MS) can be used for the study of metabolites (Kell *et al.*, 2005).

Metabolomics has been used to study several bacterial species *in vitro* under different conditions and in a complex environment such as the human gut (Aguirre *et al.*, 2014; de Graaf *et al.*, 2010). Another study of *S. aureus* showed differences in metabolites between the planktonic state and the biofilm mode of growth. In particular, specific markers of biofilm cells were found to be uptake of amino acids and a shift to cell-wall synthesis (Ammons *et al.*, 2014). Together with the other 'omics' methods, new knowledge may be acquired for novel strategies in the future combat against biofilm infections.

3.1.3.5 Staphylococcus aureus gene expression *in vivo*

In paper 4 (Larsen *et al.*, Draft)[Paper 4] a guinea foreign-body infection model was used (Figure 13) with minor modifications in relation to (Zimmerli *et al.*, 1982, 1984).

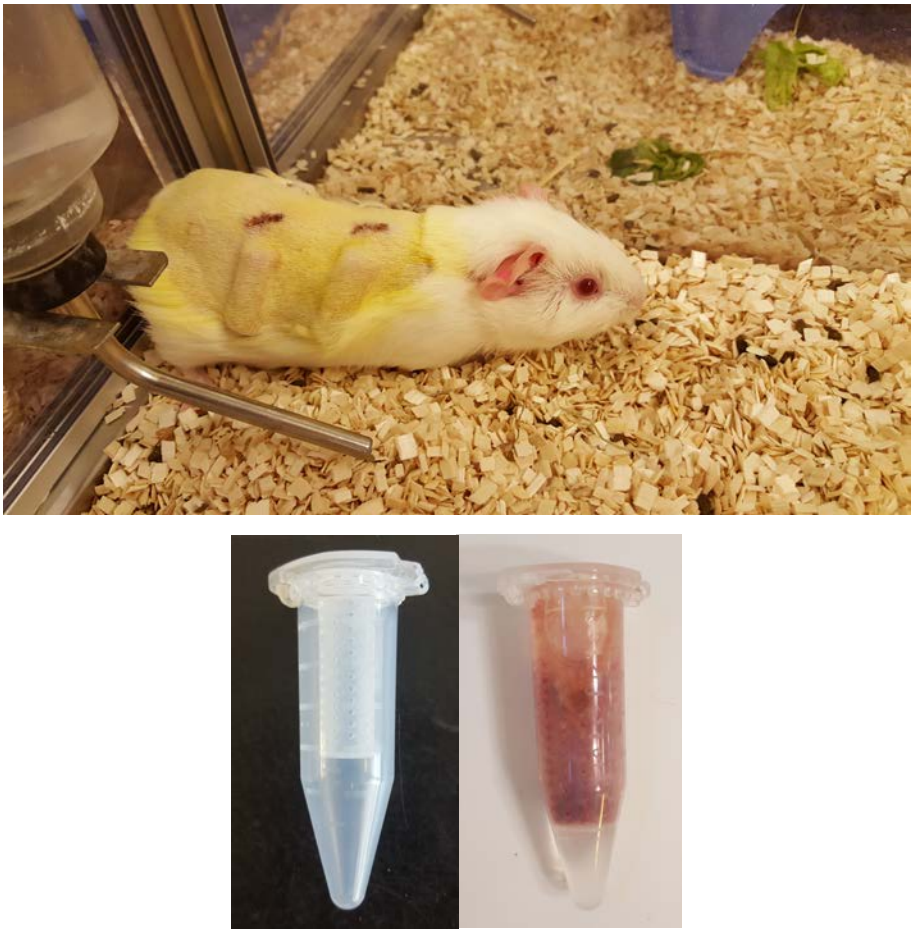


Figure 13. Foreign-body infections model in guinea pigs (top) (modified from Zimmerli et al., 1982, 1984). The guinea pig (female albino guinea pigs, Lidköbing Kaninfarm, Sweden) had subcutaneous implantations of four polytetrafluoroethylene (Teflon) cages (32mm X Ø 10 mm) perforated with 130 spaced holes, Ø 1mm) (Angst-Pfister AG, Zurich, Switzerland) in the flanks closed by intercutaneous stitches. Bottom left: Tissue cage before implantation, Right: Tissue cage after removal and submerged in RNAlater® (Ambion™, USA). Pictures copyright © Lone Heimann Larsen

The choice of the guinea pig animal model was discussed in section 1.9.1 with focus of this model's ability of to better mimic the long-term implant-related infection than a mouse or rat model. Traditionally, *S. aureus* superficial wound infections have been studied in guinea pigs, but also implant-related models are

established resembling orthopaedic infections and endocarditis (Fischer *et al.*, 1996; Maurin *et al.*, 1997; Taylor & Lee, 2012). The use of the implant-related model gave us the opportunity to investigate the gene expression profile with the more advanced RNA-Seq technique. Investigation of expression of single virulence genes in *S. aureus* has previously been done with success in this model (Goerke *et al.*, 2001; Senn *et al.*, 2005). One limitation to investigation of infections in guinea pigs is intolerance to antibiotics such as penicillin, ampicillin, erythromycin, and clindamycin due to a unique combination of intestinal flora with many Gram-positive bacteria (Hargaden & Singer, 2012). Therefore, the choice of treatment was moxifloxacin as being relevant to treatment of *S. aureus* infections internationally (ref.;Henrik C. Schønheyder, personal communication) (Bogner *et al.*, 2013), which also have been shown to have an relevant tissue concentration of 10 mg/mL in guinea pigs one hour after administration per oral (Holzgrefe *et al.*, 2014).

A *S. aureus* biofilm was established by directly injection of $2x10^3$ CFU into the tissue cages. After 3 days of infection (INF3) fluid was aspirated from the cages for either culture to confirm the infection or for transcriptomics. One group of six animals received moxifloxacin orally for four days and another group (six animals) did not receive any treatment. The animals in the moxifloxacin group were sacrificed on day 14 (MOX14), while animals in the non-treated group were sacrificed for ethical reasons on day 9 (INF9) as the infection was more aggressive than expected. Both cage fluid and the cages were saved either for direct culturing or for transcriptomics analysis (for further details see paper 4: Larsen *et al*. Draft).
The gene expression profiles showed distant relations to gene expression profiles from *in vitro* culture in LB medium and were closely related to the PJI case study by Xu et al. 2016 (Figure 14) *(In vitro* and PJI raw data are kindly provided by Xu *et al.* 2016b). A similar observation has been made for abscesses in humans and mice

(Date *et al.*, 2014). Among the *in vivo* gene expression profiles, the INF9 was the closest match to the PJI case, whereas MOX14 resembled INF3.

Focusing on the *in vivo* guinea pig infections, the profiles showed genes encoding the transcription process *rpoB, rpoC* and elongation process *fusA, tufB* were expressed in high numbers in all three groups.

dnaK was also highly expressed in all groups; the gen encodes a co-chaperone which is involved in protein folding and especially decreases aggregation of non-native proteins in the cell and thereby shields the bacteria from intermolecular aggregation (Mayer & Bukau, 2005).

Whereas the transformation of pyruvate into Acetyl-CoA and formate (*pflB* gene, highly expressed in all groups) is an anaerobic process, other genes resembling an aerobic pathway were expressed, i.e. the membrane proteins encoded by *cyoB* and *qoxA*, coding for cytochrome *bo* oxidase, a proton pump which contribute to the generation of a proton motive force via reduction of oxygen to water (Puustinen *et al.*, 1991). Overall the general picture of the metabolic response of to the environment revealed an energy gaining strategy coupled to anaerobic pathways (Fuchs *et al.*, 2007) and regulatory responses dealing with an unfriendly environment.

In contract, it was only in the newly established infection (INF3) and after moxifloxacin treatment (MOX14) that the genes for the immunoglobulin-binding, multifunctional proteins *spa* and *sbi* are highly expressed and probably contributes to evade the host immune response (Atkins *et al.*, 2008). Conversely, the genes *isaA* and *atl* encoding for autolysins are highly expressed both in INF3 and INF9. These two genes encode enzymes cleaving peptidoglycan, a process that is highly active in the exponential phase of growth and they are continuously expressed in the stationary phase (Resch *et al.*, 2005). The MOX14 group has a high expression of *ldh*, S-lactate dehydrogenase whereas the INF9 expressed *ldhD,* R-lactate dehydrogenase. This indicate lactate as a source for converting NAD^+ to NADH,

maybe also from the the host, which correlates to the lactate permease (*lctP*) that are up-regulated in MOX14.

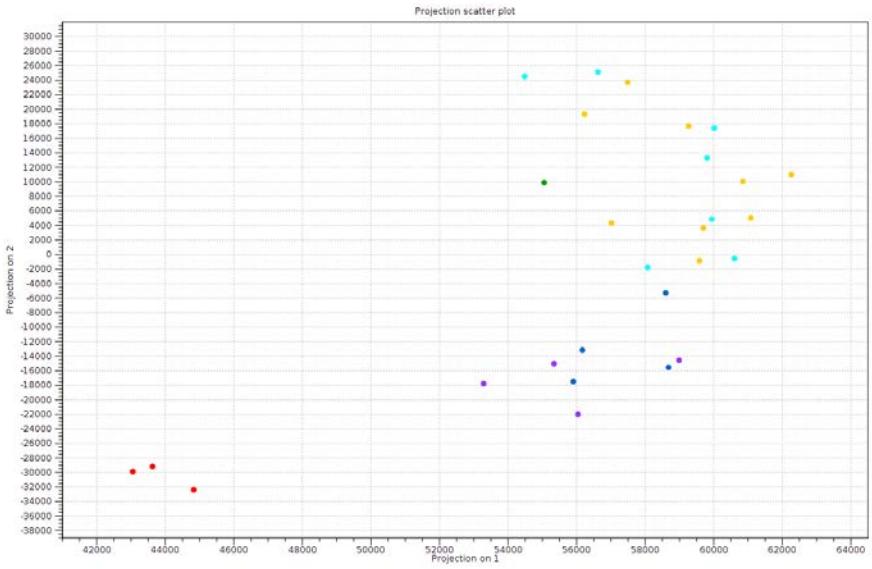
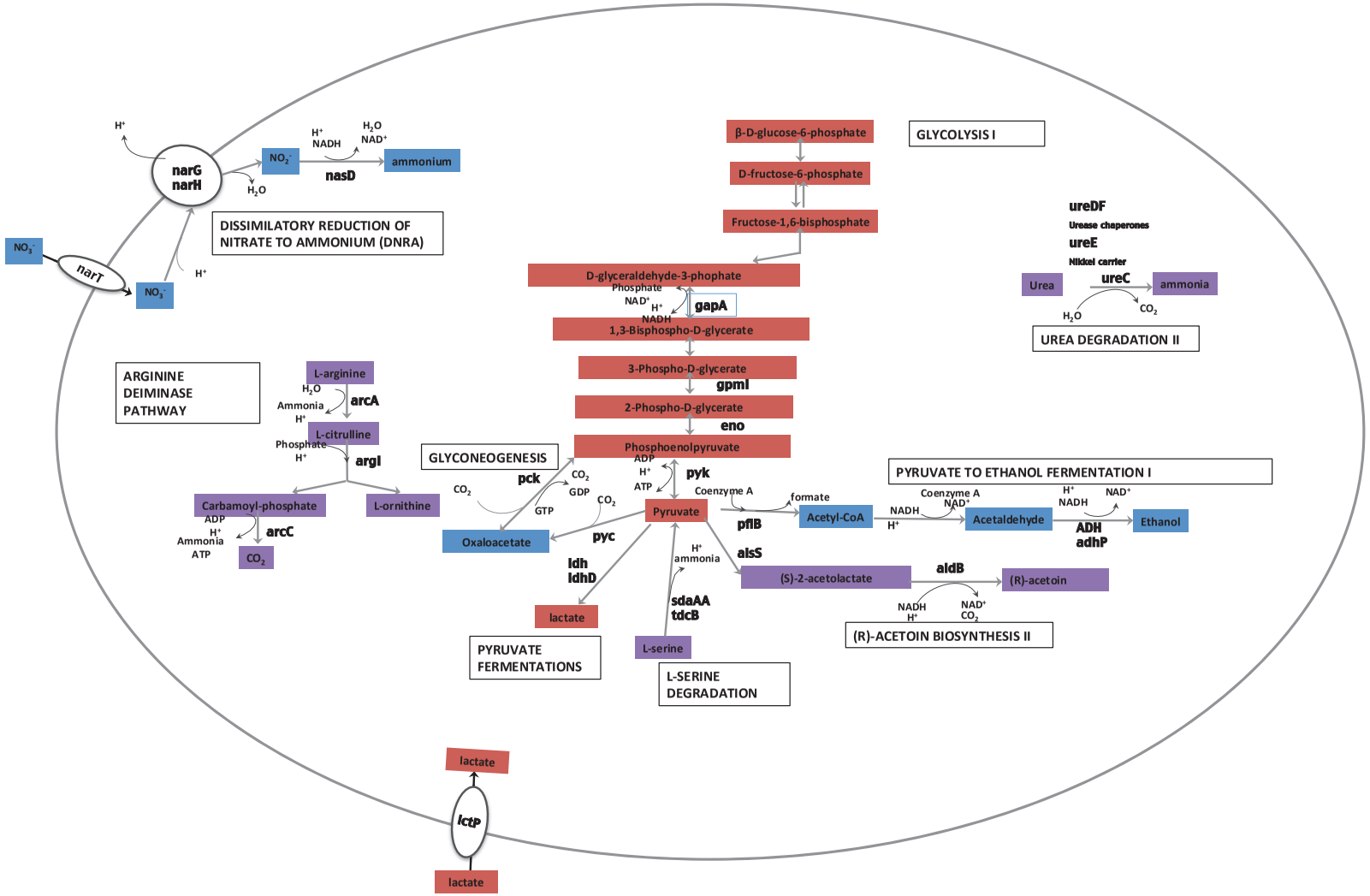


Figure 14. Principal component analysis of all the gene expression profiles. INF3, INF9 and MOX14 refer to different time points and exposure in the guinea pig model. Blue: INF3. Yellow: INF9. Light blue INF7. Purple: MOX14. Green: Human PJI. Red: *in vitro* S. aureus culture in LB media (Larsen *et al.*, Draft)[Paper 4].

In the MOX14 group the dissimilatory reduction of nitrate to ammonium (DRNA) pathway was expressed by *narG, narH, nreC, narT*, and *nasD* genes (Schlag *et al.*, 2008), whereas in the INF9 the acetoin biosynthesis was highly expressed by *als* and *aldB* are (Booth & Kroll, 1983) (Figure 15). Moreover, genes involved in urease activity *ureABC* were all highly expressed, too, and contribute to increase pH possible caused of e.g. the productions of ethanol or the accumulation of lactate in the anaerobic environment (Korem *et al.*, 2010; Resch *et al.*, 2005).



Arginine degradation V (arginine deiminase pathway)				
arcA	12	1	10	≤0.05
argI	1	22	30	>0.05
arcC	5	4	18	≤0.05
arcR*	6	0	7	≤0.05

(R)-acetoin biosynthesis II				
alsS	336	0	46	≤0.05
aldB	38	0	14	≤0.05

Urea degradation II				
ureC	34	2	32	≤0.05
ureE*	111	2	16	≤0.05
ureD*	59	2	23	≤0.05
ureF*	134	2	18	≤0.05

L-serine degradation				
tdcB	-1	81	67	≤0.05
sdaAA	9	2	12	≤0.05

Glycolysis I				
gapA	-4	3	15	≤0.05
gpmI	2	30	101	>0.05
gpmA	1	18	29	>0.05
eno	2	61	164	>0.05
pyk	1	89	98	>0.05

Pyruvate fermentation				
ldh	-16	116	6	≤0.05
ldhD	4	4	24	≤0.05
lctP	-4	50	15	≤0.05

Glyconeogenesis				
pyc	-3	51	17	≤0.05
pck	-3	20	8	≤0.05

Pyruvate to ethanol fermentation I				
pflB	-2	570	377	≤0.05
pflA*	-2	191	122	≤0.05
ADH	-2	71	34	≤0.05
adhP	-2	89	39	≤0.05

DRNA				
narG	-5	102	16	≤0.05
narH	-5	36	7	≤0.05
nasD	-3	50	23	>0.05
narT	-8	111	16	≤0.05
nreC	-3	14	4	≤0.05

Figure 15. Metabolic pathways differentially expressed in the INF9 (purple) and MOX14 group(blue) and the commonly expressed pathways (red). Pathways are named according to the MetaCyc database. In the bottom, under each pathway the fold change between the INF9 (positive) and the MOX14 (negative) are listed, the second collum shows the normalised total gene reads for MOX14 and the third shows the normalised mean reads for INF9 and the fourth shows the false discovery rate (Larsen *et al.*, Draft)[Paper 4].

Despite the different courses of infection in the guinea pig groups the expression profiles were remarkably similar and we found few genes to be differentially expressed. The metabolic pathways were characteristic for an anaerobic environment to which the bacteria adapted by regulating the pH. In general, pathways involving pyruvate metabolism were active and maybe this can be a vantage point for in future treatment strategies for *S. aureus* infection

Chapter 4. Summaries of papers 1-4

Paper 1
Optimizing culture methods for diagnosis of prosthetic joint infections: a summary of modifications and improvements reported since 1995.
Lone Heimann Larsen, Jeppe Lange, Yijuan Xu and Henrik C. Schønheyder
J. Med. Microbiol. (2012) 61, 309–316.

We did a literature review to provide an overview of improvements made in culture-based diagnostic for PJI from 1995-2012. The focus was on transport media, specimen types, culture media, and the incubation periods. Due to heterogenicity of studies data were presented narratively.
We found evidence to support superiority of cultures obtained from the diluent after sonication of prosthetic implants in comparison with culturing tissue biopsies. Culture of synovial fluid in blood culture vials was more sensitive than intraoperative swab cultures and tissue cultures. Formal evaluation of agar media for culturing PJI specimens seemed to be lacking. The polymicrobial nature of PJIs supported the routine use of an assortment of media suitable for recovery of fastidious, slow-growing, anaerobic and sublethally damaged bacteria. A number of studies supported an incubation period for up to 14 days. As a conclusion we found that culturing remains an important means to identify and characterise pathogenic microorganisms and supplements the increasing number of culture-independent assays.

Paper 2
”All in a box” a concept for optimizing microbiological diagnostic sampling in prosthetic joint infections.
Lone Heimann Larsen, Yijuan Xu, Ole Simonsen, Christian Pedersen, Henrik C. Schønheyder, Trine Rolighed Thomsen, and PRIS study group
BMC Res. Notes, (2014) 7, 418.

At the outset of the PRIS project strict principles were laid out to manage multiple sampling in PJI patients, based on experience from a pilot study. The experience

gained during the project we published the specimen logistic, as it can be useful both for research and clinical practice. Several different surgeons at different hospital did the sampling and still we managed to obtain high completeness of our specimen sets (approx. 90%). The paper emphasised the need to standardise the pre-analytical phase in order to make valid comparisons of different specimen types and diagnostic methods.

Paper 3
Diagnostic value of culture and 16S r*RNA* sequencing in patients undergoing revision surgery for infection of a hip or knee arthroplasty.
Lone Heimann Larsen, Vesal Khalid, Yijuan Xu, Trine Rolighed Thomsen, Henrik C. Schønheyder and The PRIS Study group
In prep.

Within the framework of the prospective multidisciplinary study PRIS enrolling patients with hip or knee prosthetic failures, we assessed the diagnostic contribution of different specimen types for PJI and whether 16S *rRNA* sequencing added significant information to culturing. The ultimate goal was to define an optimal specimen set. 110 patients underwent 114 revisions ('cases') where multiple specimens of each type (joint fluid, biopsies of bone and soft tissue, swabs from the prosthesis) were obtained besides prosthetic component(s) if removed. Additionally, a set of 5 soft tissue biopsies was obtained according to standard practice. Culturing was done for 14 days, and 16S *rRNA* sequencing was performed at end of study. A diagnosis of PJI was made in 42 cases: 39 were culture positive according to standard criteria; three cases had an entirely clinical diagnosis, whereas one additional case was later confirmed by sequencing. We found the optimal specimen set to include joint fluid, five soft tissue biopsies, and prosthetic components. In aggregate, positivity of the optimal set concorded well with the PJI diagnosis (93%; 39/42) despite a lower concordance for individual specimen types. In the light of an optimised culture diagnostic, the significant contribution of 16S *rRNA* sequencing were less importance and must only be applied on selected patients with low-term chronic cases.

Paper 4
Staphylococcus aureus gene expression profiles in a guinea pig biofilm infection model: Effect of time and antibiotic treatment with moxifloxacin.
Lone Heimann Larsen, Yijuan Xu, Kåre L. Nielsen, Henrik C. Schønheyder and Trine Rolighed Thomsen
Draft

We established a *S. aureus* foreign-body infection model in guinea pigs. Specimens were taken after 3 days (INF3), and subsequently one group was treated orally with moxifloxacin for 4 days, and a second group did not receive treatment. The treated group was sacrificed at day 14 (MOX14), while the untreated group was sacrificed on day 9 (INF9). The gene expression profiles were analysed for all tree groups and were remarkable similar despite the different course of infection. The gene expression profiles also concorded well with the human PJI infection being the source of same strain. The data shows anaerobic pathways to predominate and pyruvate metabolism in particular. Different strategies were activated in order to handle the acidic environment, and this may be a vantage point for future treatment strategies for *S. aureus* infection.

Chapter 5. Conclusions and perspectives

Case: In 1994 a patient had a total knee replacement, followed shortly by an insertion of a cemented patella component. Throughout the years he had continuously pain and in 2005 he contacted the hospital again with severe pain, instable prosthesis. The diagnostic imaging revealed synovitis, which was deemed to be related to an on going rheumatologic disease. A revision was carried out with replacement of the prosthesis but the patella component was retained.

The culturing revealed coagulase-negative staphylococci in 3 out of 5 synovial biopsies and the patient was put on treatment with antibiotics.

After the revision the patient recurrent stress-related pain, swelling of the knee and a slight, but persistent elevation of inflammatory markers. Repeated joint aspirations in 2006 were negative on standard culture. The patient was treated with a brief course of steroids.

In 2012, the patient had persistent stress-related pain and swelling of the knee. The patient was included in the PRIS project and advanced diagnostic nuclear imaging showed a 'hotspot' at the interface between the lateral tibia plateau and the prosthesis. The prosthesis was removed and submitted to extensive microbiological diagnostics. The microbiology showed *S. epidermidis* with an antibiogram similar to that reported in 2005, making it very likely that a chronic biofilm prosthesis infection had persistent for the last 7 years. The patient was treated with antibiotics and the infections parameter was normalised for the first time since 1994.

Larsen et al. Eurobiofilm 2013, Genth, Belgium (Larsen *et al.*, 2013)

The above-mentioned case is one example of the importance of improvement of our knowledge related to PJI and to improve the diagnosis to avoid long-term painful courses of illness.

To improve the diagnosis and treatment of PJI, the first priority is to recognise the microorganism/s by using the best combination of specimens and diagnostic methods. No single specimen type or diagnostic method has yet been proven to cover the entire variety of PJI patients. Secondly, new treatment strategies are warranted, especially for the biofilm related infections. A better understanding of how microorganisms behave in biofilm infection may contribute to revelling points of interest. Therefore, one of the aims of this PhD project was to characterise the clinical diagnostic challenge of PJI and to evaluate well-established diagnostic methods together with explorative methods, for investigation of the microbial diversity in implant-related infections. Moreover, the work with the guinea pig biofilm infection model and transcriptomics would provides insights into the S. aureus biofilm infections *in vivo* and especially where the biofilm infections may be most vulnerable for therapeutic interventions, the metabolistic pathways which are vital for the bacterial survival in a hostile host environment.

Biofilm infections are difficult to diagnose due to the adherence of bacteria to the implant and the implant needs to be removed for an identification of the microorganism and diagnosis. Failure to detect biofilm bacteria can have significant consequences. Undiscovered bacteria either caused by polymicrobial infections or false negative diagnostics can lead to only partial coverage by antibiotic. When the treatment are chosen in accordance with results obtained by standard cultures and it may even lead to mistaking a PJI for aseptic failure. Besides consequences for the individual patient, such mistakes may lead to biased assessment of prosthetic failures overall. Thus, surgical intervention serves the dual purpose of providing

diagnostic specimens from relevant sites and to clear out recalcitrant biofilm infections. To have the relevant specimens is crucial for the optimal diagnosis, and we tested a logistic concept with several different specimen types in the relevant clinical settings and managed to receive 90% of all planned specimens. This concept was the foundation for the subsequent evaluation of which bacteria were present in the PJIs. A second obstacle for the diagnosis of PJI is the low growth rate of bacteria in biofilm and the need to 'awake' the persisters, requirements that are not necessarily met by standard culture methods. The standard method for culture-based diagnostic is soft tissue biopsies both in Denmark and internationally as outlined in recent international guidelines. However, since 1999 the use of sonication diluents from prosthetic component has been shown to increase the diagnostic sensitivity of culture methods. A part of this contribution is awakening of persisters in the biofilm. Even though new specimen types show promising results, no single one has been proven to cover all PJI patients. Thus, the optimal microbial diagnosis of PJI requires a combination of specimens and methods rather than a single specimen and method at present. We showed that a specimen collection of joint fluid, multiple soft tissue biopsies, and the prosthetic component fulfils this task and is superior to the time-honoured reference standard of multiple tissue biopsies. The merit of the specimen set is expedience and accuracy of diagnosis, and it overcomes the heterogeneous distribution of biofilms in PJI.

Our optimised culturing with a broad range of media and prolonged incubation times was superior to molecular methods in cases with acute infection with easily culturable bacteria and can provide a preliminary diagnosis within one – two days. But for the more challenging cases suspected of chronic infection, the use of molecular techniques based on PCR and sequencing can supplement results obtained by culture and are likely to provide a faster diagnosis in optimised diagnostic settings.

The uses of molecular techniques have shown promising results in the literature, and especially multiplex PCR has proven to contribute to the diagnosis of PJI, but only in research settings. Recently, a large multicentre study concluded that the use of PCR based diagnostics did not contribute significantly to the diagnosis of PJI used on all types of patients. These results are in line with the results from the PRIS study reported in this thesis, we suggested that molecular technique shut be restricted tor a special group of patients until more effective methods are developed for the diagnosis of PJI. For the time being molecular techniques can be reserved for patients in whom the diagnosis might be challenging and for specific specimen types.

Being a relatively young field of research, many unresolved questions exist regarding the pathogenesis and colonisation strategy of the bacteria involved. Werner Zimmerli has pointed out that spontaneously healing of a implant-related infection has never happened – so "knowing the enemy" must be one of the next steps for finding the possible weak spots for an offensive. Experience from the clinical setting and laboratory experiments need to be supplemented with *in vivo* experiments in order to get more insight into the world of biofilm and to disentangle the complexity. A crucial point is to analyse the behaviour on a molecular level, hopefully identifying backdoors for eliminating implant-related infections without a surgical intervention in the future.
The uses of a model system have to be chosen carefully in respect to the question together with the approaches used for analysis. The different 'omics' approaches can be applied, each gives insight into different levels of the metabolic possibilities/activities in infection, these techniques can help to answer some of the questions and help enlighten some of the complex interactions.
The use of transcriptomics for the study of an *in vivo* model resembling an implant-related infection gave us insights into some of the complex regulatory mechanisms in a *S. aureus* infection and the regulation and adaptation to the anaerobic and

acidic environment, confirming findings in a pilot study of a human PJI. The discovery of highly expressed metabolic pathways centred around pyruvate metabolism and different strategies are applied for regulation of pH, which gave us points of interest. With further investigation of infections *in vivo* and better understanding of metabolic regulation together with the already published knowledge regarding virulence factors and biofilm control, this might lead us to new targets in the fight against microorganism and the biofilm form of life.

In the dream setup for the diagnosis and treatment of PJI patients, surgical intervention would be obviated. Treatment would eradicate the infecting microorganisms irrespective of biofilm formation. Still, there is a long way to go, but progress in a smaller scale would be welcome. With an improved diagnosis of PJI and an increasing understanding of the pathogenesis in the bacterial biofilm, better treatment might be one step closer.

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Appendix A. Paper 1

Larsen, L.H., Lange, J., Xu, Y., and Schønheyder, H.C. (2012). Optimizing culture methods for diagnosis of prosthetic joint infections: a summary of modifications and improvements reported since 1995. J. Med. Microbiol. 61, 309–316.

Journal of Medical Microbiology (2012), **61**, 309–316 DOI 10.1099/jmm.0.035303-0

Review

Optimizing culture methods for diagnosis of prosthetic joint infections: a summary of modifications and improvements reported since 1995

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Improving diagnosis of prosthetic joint infections (PJIs) has become an increasing challenge due to a steadily rising number of patients with prosthetic implants. Based on a systematic literature search we have ascertained the evidence base for improvement of culture diagnosis. We searched PubMed/MEDLINE using the medical subject heading (MeSH) 'prosthesis-related infections' 1995 through 2010 without further restrictions. An analogous search was conducted for ISI Web of Knowledge. A total of 1409 reports were screened for original results, obtained by methods described in sufficient detail to make replication possible. We gave priority to methods for sample preparation, culture media, culture methods and incubation time. Clinical sensitivity and specificity were calculated where possible. We found evidence to support superiority of cultures obtained from the diluent after sonication of prosthetic implants in comparison with culturing tissue biopsies. Sonication parameters and accessory steps have been studied extensively, and thresholds for significant growth have been defined. Conversely, methods for processing of soft tissue biopsies have been studied to a limited extent. Culture of synovial fluid in blood culture vials has been shown to be more sensitive (90–92 %) than intraoperative swab cultures (68–76 %) and tissue cultures (77–82 %). Formal evaluation of agar media for culturing PJI specimens seemed to be lacking. The polymicrobial nature of PJIs supports the routine use of an assortment of media suitable for recovery of fastidious, slow-growing, anaerobic and sublethally damaged bacteria. A number of studies supported an incubation period for up to 14 days. Although we identified evidence-based improvements of culture methods, there is a need for more studies especially with regard to tissue biopsies. Culturing remains an important means to identify and characterize pathogenic micro-organisms and supplements the increasing number of culture-independent assays.

Introduction

Joint replacement has become one of the most common surgical procedures in industrialized countries and contributes significantly to the mobility and quality of life of elderly people. Even with the best precautions, prosthetic joint infections (PJIs) do occur and they have become a significant burden on orthopaedic services due to the sheer number of patients with hip and knee prostheses. The clinical spectrum of PJIs is variable and includes both overt and silent infections, and bacterial pathogens are envisaged to have a role in 'aseptic' loosening (Zimmerli *et al.*, 2004; Zappe *et al.*, 2008; Pedersen *et al.*, 2010).

For these reasons, diagnostic methods have received increasing attention. A range of molecular techniques have been introduced primarily as research tools and a new concept of PJIs is gradually emerging (McDowell & Patrick,

A supplementary table is available with the online version of this paper.

2005; Achermann *et al.*, 2010). Bacteria common in PJIs are typically organized in a biofilm, which is a microbial community enclosed within an extracellular matrix (Donlan, 2002). The normal microbiota of the skin is the most common source of bacteria detected in PJIs, and 16S rRNA gene sequence-based methods have revealed infections to be polymicrobial in line with results achieved by standard culture methods in some studies (Tunney *et al.*, 1999; Moojen *et al.*, 2007).

Despite the increased utilization of molecular techniques, culture methods are indispensable for determination of antibiotic susceptibility and they are an important means of confirming results obtained by culture-independent methods. Kamme & Lindberg (1981) were the first to report separate sampling and processing of multiple tissue biopsies taken in proximity to hip prostheses as a means of increasing the accuracy of infection diagnosis. Over the years, a number of studies have addressed different methodological issues that may have an impact on the yield of positive cultures. However, to our knowledge, few attempts have been made to systematize and critically assess such methods (Gollwitzer *et al.*, 2006).

Several overviews of the biochemistry, clinical diagnosis and treatment of PJIs have recently been published (Senthi *et al.*, 2011; Gomez & Patel, 2011a, b). Still, there is a need for a more detailed assessment of methods for sample preparation, culture media, culture methods and incubation time. The aim of this review was to determine the best practices for improvement of culture diagnosis of PJIs.

Methodological approach

A search of ISI Web of Knowledge and the US National Library of Medicine's MEDLINE database was conducted for relevant articles. Access to MEDLINE was through PubMed, using the medical subject heading (MeSH term) 'prosthesis-related infections' (introduced 1992) with the subheading 'microbiology' and a restriction to the years 1995–2010 (date of search 15 January 2011). No restriction was made to orthopaedic infections because methodological studies addressing other prosthetic infections were seen as potentially relevant.

A total of 1409 articles were screened initially by title and subsequently by the contents of the abstract and the section on material and methods. We specifically sought information on modification, improvement or optimization of diagnostic methods as well as detailed descriptions of sample preparation and culture methods, including incubation time. Further references were obtained from reference lists.

For each eligible article, we collected the following information: year of publication, researcher/research group, design (observational study vs clinical trial, prospective vs retrospective study, comparative vs non-comparative study), diagnostic criteria for PJI, method(s) evaluated, reference method ('gold standard') if any, unit of observation (patients, samples, bacterial isolates), report format (cross tables or aggregated figures) and accuracy [sensitivity, specificity, positive and negative predictive values (PPV and NPV)].

Few studies did fulfil basic criteria for a systematic review. The most pertinent problems were lack of a gold standard and independent evaluation of results. Statistical analyses of results were often unclear and units of observation differed between (and sometimes within) studies. Where possible, we assessed test performance based on numbers of patients, and we defined the sensitivity of a given method as the number of PJI patients with a positive culture divided by the number of PJI patients examined by that method. Likewise, specificity was defined as the number of patients without PJI who had a negative culture divided by the number of non-PJI patients examined by the method. In order to compare the accuracy of methods, we calculated exact 95 % confidence limits for proportions [(#;#)] (Stata 11, College Station, Texas, USA).

Current approaches

Transportation systems

We found no studies evaluating the performance of transport media with orthopaedic samples. A non-comparative study used broad-necked containers with Stuart transport medium for surgical biopsies from patients undergoing prosthetic joint revision (Mikkelsen *et al.*, 2006). However, several studies evaluated the performance of transport media with cultures of fastidious and robust aerobic and anaerobic bacteria according to the M40-A standard (Clinical and Laboratory Standards Institute) (CLSI, 2003; Rishmawi *et al.*, 2007; Van Horn *et al.*, 2008; Stoner *et al.*, 2008; Tano & Melhus, 2011). Performance differed depending on temperature, holding time and bacterial strains. In general, good preservation was reported for media held at 4 °C but the results varied at room temperature. Results obtained with simulated polymicrobial samples were less predictable (Tano & Melhus, 2011). Transport systems with Amies medium or variations thereof maintained viability better than Stuart medium, but promoted growth of some bacteria (Tano & Melhus, 2011).

Tunney *et al.* (1998, 1999) used anaerobic jars for transportation of prosthetic components from the surgical theatre to the laboratory followed by strict anaerobic processing of samples. Anaerobes accounted for a high proportion (62 %) of isolates from the prosthetic components.

Sample preparation

PJI samples comprise frank pus, purulent fluids, synovial fluid, synovia and/or other soft tissue samples, bone biopsies, prosthetic components or entire prostheses.

Different treatments are applied to the samples before inoculation takes place, either to dislodge bacteria from a matrix or to increase the density. Several studies have addressed such preparatory steps.

Synovial fluid. Techniques primarily developed for blood culturing have been pivotal for processing of synovial fluid in PJIs. Paediatric blood culture vials were reported to detect more pathogens than agar plate methods (62 vs 51 pathogens; $P=0.001$) with fewer contaminants (1 vs 11 contaminants; $P=0.006$) (Hughes *et al.*, 2001). The authors related the contaminants to handling and inspection of agar plates.

Two studies compared culturing of synovial fluid with surgical swabs or tissue biopsies in patients with PJIs using direct inoculation of aerobic and anaerobic blood culture vials for synovial fluid and agar plates for swabs or biopsies (Levine & Evans, 2001; Font-Vizcarra *et al.*, 2010). Both studies showed higher sensitivity and specificity for direct inoculation although the precision was low (Table 1).

Melhus & Tjernberg (2000) evaluated three different blood culture vials (two anaerobic and one paediatric) for recovery of anaerobic bacteria in a simulation study with 10 bacterial strains. They found differential growth of anaerobic bacteria in blood culture vials and thioglycolate broth whereas chopped meat broth (a traditional anaerobic medium) performed well.

Swab cultures. Culturing of superficial swabs from wound drainage has a traditional place in the diagnosis of deep bacterial infections (Mackowiak *et al.*, 1978). Cuñé *et al.* (2009) evaluated such cultures in patients with acute postoperative PJI and found isolation of *Staphylococcus aureus* and enteric rods highly predictive of the aetiological organism (PPV >86 %, NPV >94 %).

As shown in Table 1, intraoperative swab cultures had a lower sensitivity compared with culturing of synovial fluid and tissue biopsies according to the study by Font-Vizcarra *et al.* (2010). Of note, we did not find studies that addressed the performance of different types of swabs in orthopaedic infections.

Soft tissue samples. The literature search did not reveal comparative studies on the preparation of tissue samples for microbial cultures. Three different preparation methods were applied for tissue samples: (1) partitioning into smaller pieces with a surgical knife (Mikkelsen *et al.*, 2006), (2) grinding with a mortar and pestle or (3) stomaching.

Homogenization by use of mortar and pestle was applied in several studies (Günthard *et al.*, 1994; Levine & Evans, 2001). Günthard *et al.* (1994) reported two cases of endocarditis with negative cultures by direct plating of cardiac tissue, but positive cultures matching previous blood cultures after homogenization with the Ten Broeck tissue grinder.

The Stomacher technique was applied for PJI samples in a study addressing *Propionibacterium acnes* in particular (Butler-Wu *et al.*, 2011). The release of cultivable bacteria by this technique has recently been evaluated in food microbiology (Hannah *et al.*, 2011).

Table 1. Comparison of three culture methods for synovia and synovial fluid from patients with infected hip or knee prostheses

	No. of patients	Swab culture (intraoperative)		Synovial fluid culture (blood culture vials)		Tissue culture (sample set)	
		Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
Levine & Evans (2001)	32/34/20	76 % (19/25)	86 % (6/7)	92 % (23/25)	100 % (9/9)	77 % (10/13)	100 % (7/7)
95 % CI		(55; 91)	(42; 100)	(74; 99)	(66; 100)	(46; 95)	(59; 100)
Font-Vizcarra *et al.* (2010)	150	68 % (59/87)	99 % (62/63)	90 % (78/87)	100 % (63/63)	82 % (71/87)	81 % (51/63)
95 % CI		(57; 77)	(91; 100)	(81; 95)	(94; 100)	(72; 89)	(89; 90)

Solid samples and prosthetic components. Culturing of bone biopsies has rarely been reported in studies on PJIs. Conversely, there is an extensive literature on processing of prosthetic materials by sonication. Tunney *et al.* (1998) applied mild sonication [5 min, 50 kHz, corrected by McDowell & Patrick (2005)] to dislodge bacteria adherent to the implants and reported an improved detection of infective agents in hip PJIs. A number of subsequent studies confirmed a higher yield by this method (Table 2) (Trampuz *et al.*, 2006, 2007; Bjerkan *et al.*, 2009; Monsen *et al.*, 2009; Piper *et al.*, 2009). Sonication was evaluated in experiments with bacterial cultures relevant to PJIs, indicating, especially for Gram-negative bacteria, a trade-off between dislodgement of bacteria and decreased viability. According to Monsen *et al.* (2009), the duration of sonication and the material of the tube were critical parameters for the cultivability of bacteria after treatment. They recommended the following parameters for sonication: 40 kHz for 7 min at 22 °C in order to provide maximum effect and preserve viability of Gram-negative bacteria. No further improvement was observed by lowering the temperature. Thin-walled glass tubes provided an increased effect compared to plastic tubes. Furthermore, Monsen *et al.* (2009) compared sonication in tubes deeply submerged in the sonication bath with sonication in tubes only partially submerged (~25 %), and they found a similar effect as long as the sample in the tube was completely submerged in the diluent.

By sonication bacteria are released into the diluent, and a centrifugation step can be required for concentrating bacteria into a smaller volume. When processing entire prostheses the volume of diluent makes centrifugation of aliquots the most practical way to proceed (Monsen *et al.*, 2009; Piper *et al.*, 2009).

Newly published studies indicated that vortexing of the sample for 30 s before and after sonication may increase the yield of positive cultures (Trampuz *et al.*, 2007; Kobayashi *et al.*, 2009; Sampedro *et al.*, 2010).

In a thorough study of a patient with PJI, Sendi *et al.* (2010) observed phenotypic variation of *Escherichia coli* recovered from periprosthetic biopsies and from the diluent after sonication of the entire prosthesis. These small-colony variants (SCVs) were clonally indistinguishable from *E. coli* with normal morphological features. Cultures from synovial fluid showed colonies with the normal phenotype only. With further subcultivation the SCV *E. coli* adapted to the normal phenotype. The authors concluded that the different phenotypes most likely originated from different niches, i.e. the biofilm on the prosthesis and the synovial fluid. Proctor *et al.* (2006) did also bring attention to this issue with reference to *S. aureus* isolates surviving within mammalian cells: SCV *S. aureus* has an impaired growth rate and unusual biochemical characteristics that may hamper its correct identification.

Culture media

The media commonly used for PJI samples are non-selective and enriched with a content of blood or blood

Table 2. Comparative studies of sonication and yield of positive cultures in patients with PJIs

	No. of patients	+ Sonication (prosthesis)		− Sonication (tissue)		Comments
		Sensitivity	Specificity	Sensitivity	Specificity	
Piper *et al.* (2009)	134	67 % (22/33)	98 % (99/101)	55 % (18/33)	95 % (96/101)	PJIs of the shoulder
95 % CI		(48; 82)	(93; 100)	(36; 72)	(89; 98)	
Esteban *et al.* (2008)	31	94 % (16/17)	50 % (7/14)	88 % (15/17)	100 % (8/8)	PJIs of hip, knee and miscellaneous
95 % CI		(71; 100)	(23; 77)	(64; 99)	(63; 100)	
Trampuz *et al.* (2007)	331	78 % (62/79)	99 % (249/252)	61 % (48/79)	99 % (250/252)	PJIs of hip and knee
95 % CI		(68; 87)	(97; 100)	(49; 72)	(97; 100)	
Trampuz *et al.* (2006)	78	75 % (18/24)	87 %* (47/54)	54 % (13/24)	98 %* (53/54)	Contamination due to leakage of sonication bags
95 % CI		(53; 90)	(75; 95)	(33; 74)	(90; 100)	

*Excluding the patients who received antibiotics, the specificity was 100 % for all types of samples.

products. However, studies rarely provided detailed information on culture media. In Supplementary Table S1 in JMM Online, we have listed papers which provided sufficient details as well as a list of bacterial isolates. To our knowledge, no studies have formally evaluated the performance of different agar media in the diagnosis of PJIs.

A differential effect of the culture medium was noted by van Kats *et al.* (2010) in a study of heart valve biopsies. The transport medium was inoculated both into blood culture vials (FA and FN blood culture media, BacT/Alert; bioMérieux) and thioglycolate broth. Coagulase-negative staphylococci and *Propionibacterium* species were isolated in higher frequencies with thioglycolate broth than with blood culture vials, and vice versa for *S. aureus*. Similarly, thioglycolate broth was reported to promote growth of a broader range of anaerobic bacteria than anaerobic blood culture vials in an experimental study mentioned previously (Melhus & Tjernberg, 2000). The use of pre-reduced culture media and strict anaerobic techniques has been emphasized in some studies addressing optimization of diagnosis of PJI (Tunney *et al.*, 1998, 1999; McDowell & Patrick, 2005).

Nevertheless, not only can the type of medium have an effect on bacterial cultivability but also the viscosity impacts on the spectrum of micro-organisms isolated (Wyatt & Archer, 1988).

Incubation

The papers listed in Supplementary Table S1 reported incubation periods from 2 to 14 days and this broad range applied to both aerobic and anaerobic cultures. In the majority of studies, the incubation period was in the order of 5 days for aerobic cultures and 7 days for anaerobic cultures (Trampuz *et al.*, 2007; Piper *et al.*, 2009; Achermann *et al.*, 2010). According to Schäfer *et al.* (2008), prolongation of the incubation period was associated with an increase in the proportion of positive samples and diversity of bacterial isolates (Table 3). In line with this, Schäfer *et al.* (2008) recommended an incubation period of up to 14 days based especially on late recovery of aerobic Gram-positive rods, *Propionibacterium* species and *Peptostreptococcus* species. In the aforementioned study by Günthard *et al.* (1994), growth of *P. acnes* was reported to require more than 8 days, and, similarly, Butler-Wu *et al.* (2011) found a 29 % increase in cultures positive for *P. acnes* when comparing a 13 day incubation period with 7 days. Likewise, an incubation period of 14 days was used by Sendi *et al.* (2010) for recovery of SCV *E. coli*.

Skovby *et al.* (2011) described a practical scheme for extending the incubation of PJI cultures beyond day 5 by subculturing of visually negative semi-solid thioglycolate media onto appropriate agar plates, which were evaluated on day 12. The scheme resulted in a 10 % increase in bacteriological findings deemed to be clinically significant.

Quantitative aspects of cultures

In their study on multiple biopsies in prosthetic hip infections, Kamme & Lindberg (1981) were primarily concerned with contamination during sampling, transportation and handling in the laboratory. The biopsies (n=5) were taken from the same area adjacent to the cement or prosthesis guided by suspicion of infection or bone resorption. In 10 of 31 control arthroplasties, one or two biopsies were positive per set [*P. acnes* accounted for 9 (69 %) of 13 bacterial isolates]. On this basis, the authors defined $\geqslant$three positive biopsies as significant growth. To our knowledge, this criterion has only been evaluated once, namely in a retrospective Danish study comprising 118 patients with knee prosthetic joints [specificity 100 % (94; 100); sensitivity 46 % (27; 67)] (Mikkelsen *et al.*, 2006).

Atkins *et al.* (1998) undertook a prospective evaluation of microbiological diagnostic criteria of PJIs and found growth of an indistinguishable micro-organism from cultures of at least three independent specimens (biopsies and synovial fluid included) to be highly predictive of infection (sensitivity 65 %, specificity 99.6 %). A caveat to this study was the fact that the sampling strategy varied somewhat between patients. By mathematical modelling, $\geqslant$five specimens was found to be the optimal number of samples in order to minimize false-negative outcomes.

In accordance with the two former studies, Schäfer *et al.* (2008) obtained sets of five biopsies of both periprosthetic tissue and the synovial membrane in PJIs (hips and knees included). They deemed $\geqslant$two biopsies with indistinguishable growth to indicate infection, but made a concession to one positive culture in cases with acute inflammation diagnosed by histopathological examination.

A different aspect of multiple samples was highlighted by Zappe *et al.* (2008), who investigated the role of *Propionibacterium* species in PJIs. The study analysed eight patients who formed a subset from a larger study, and all but one had multiple positive samples (median proportion of positive samples 39 %, interquartile range 18–55 %). The mean number of biopsies was 9.5 in cases with positive

Table 3. Proportion of samples with early and late growth during prolonged incubation

	No of samples; patients	Incubation period (days)	Early detection of growth ($\leqslant$7 days)	Late detection of growth (>7 days)
Schäfer *et al.* (2008)	S: 284; P: 110	14	73 %	27 %
Butler-Wu *et al.* (2011)	S: 557; P: 173	13	71 %	29 %

cultures and the authors stressed the importance of multiple samples, especially for patients treated with antibiotics before surgery.

Towards more efficient diagnostics

Review of the literature has identified a number of options for improvement of culture diagnosis in patients with PJI. During the last 15 years, there has been a growing understanding of the role of polymicrobial infection and biofilm formation in PJIs and this has been a challenge to time-honoured culture methods. The causative microbiota originates predominantly from the skin, and many of these micro-organisms show different phenotypes with varying cultivability (Donlan, 2002; Proctor *et al.*, 2006).

The key areas for evidence-based improvements were the following:

- collection of multiple samples from the site of infection;
- selection of the transportation system;
- inoculation of synovial fluid directly into blood culture vials;
- culture of sonication diluent after sonication of prosthetic components if necessary combined with a centrifugation step;
- prolongation of incubation of cultures for up to 14 days.

The crucial number of positive specimens is still debatable as Kamme & Lindberg (1981) and Atkins *et al.* (1998) both settled for three independent specimens to confirm a diagnosis of PJI, but Schäfer *et al.* (2008) required only two.

The importance of transportation of specimens from the operation room to the laboratory seems to have been underestimated but so far surrogate studies with bacterial cultures have indicated Amies medium and variations thereof to be reliable within a time frame of 24 h (Rishmawi *et al.*, 2007; Van Horn *et al.*, 2008; Stoner *et al.*, 2008; Tano & Melhus, 2011). A cool (4 °C) transport chain may not be vital as the studies quoted showed acceptable preservation of viability at room temperature.

An effective protocol has been devised for sonication of prosthetic material (Fig. 1) and significant growth has been defined in terms of c.f.u. per entire implant or per volume of sonication diluent.

The direct inoculation of synovial fluid in blood culture vials has been proven to increase rates of positive cultures deemed clinically significant and to reduce contamination as compared with plate cultures (Hughes *et al.*, 2001). However, the optimal choice of blood culture medium and aerobic versus anaerobic conditions is open for discussion (Melhus & Tjernberg, 2000).

In a review of culture techniques for biofilms, Høgdall *et al.* (2010) emphasized that many bacteria may be inactive,

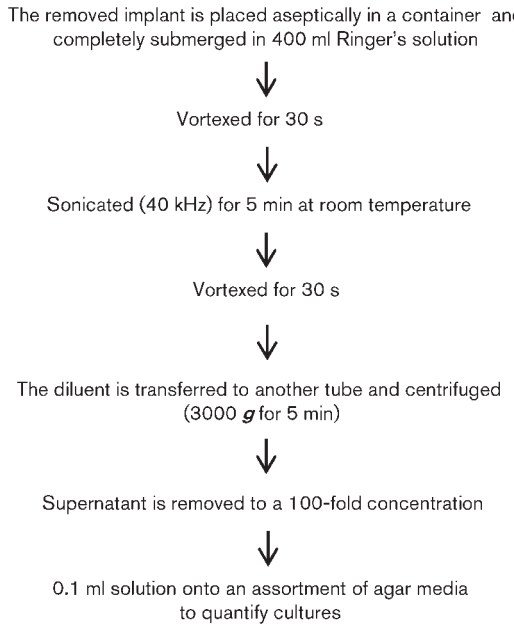

Fig. 1. Effective protocol for sonication of prosthetic material. Limits for interpretation of growth: ⩾20 c.f.u. per plate [⩾20 c.f.u. (10 ml Ringer's solution)$^{-1}$]. Ringer's solution was used by Trampuz *et al.* (2007), Monsen *et al.* (2009) and Sampedro *et al.* (2010). Ringer's solution (25 %, v/v) containing L-cysteine (0.05 %, w/v) as a reducing agent for optimal isolation of *P. acnes* was used by Tunney *et al.* (1998, 1999). According to Tunney *et al.* (1998, 1999), Trampuz *et al.* (2007), Monsen *et al.* (2009), Sampedro *et al.* (2010), Gomez & Patel (2011b).

dormant or damaged by sonication during sample preparation. The time range until colonies can be recognized on agar plates can therefore be very prolonged (Høgdall *et al.*, 2010). These observations are compatible with an incubation period of up to 14 days as suggested by both Schäfer *et al.* (2008) and Butler-Wu *et al.* (2011).

Conclusions and perspective

Only a few diagnostic studies on PJIs fulfilled rigid criteria for a systematic review. The most pertinent problems were lack of an independent reference standard and inclusion of patients because their samples had been processed with the evaluated method (STARD Statement 2008). 'Best evidence' was therefore based primarily on concordant results from different researchers. The robustness and accuracy of methods coming from the research laboratory should be confirmed in the routine clinical setting before they become standard practice. With the increased handling of PJI samples, one of the concerns is the increased risk of contamination. Various precautions can be taken including handling of samples and cultures in a laminar air flow bench (Schäfer *et al.*, 2008), separate incubators for prolonged incubation and, in general, keeping inspections to a minimum.

In future studies, it will be important to correlate results obtained by culture with those obtained by culture-independent methods (Tunney *et al.*, 1999; Panousis *et al.*, 2005; McDowell & Patrick, 2005; Achermann *et al.*, 2010) and thereby possibly define a new reference standard.

Techniques for processing of biopsies of soft or solid tissue should be studied in the same diligent way as sonication of prosthetic implants, as the studies showed that the effective dislodgement of bacteria can increase the sensitivity. Tissue grinding has proved useful for other areas of microbiological research (Günthard *et al.*, 1994) and should be tested preferably with single use equipment.

The time-honoured concept of broad-range bacteriological media seems still to be valid considering the multiple bacterial species associated with PJI. Whether supplementary or new media can augment the yield of cultures is not clear. The polymicrobial nature of many PJIs makes control of overgrowth by, for example, coagulase-negative staphylococci an important consideration. The use of selective media must be given due consideration.

As mentioned briefly, the viscosity of the medium may have an impact on the culturability of planktonic and biofilm-adapted bacterial phenotypes. This speaks in favour of semi-solid media such as thioglycolate agar and semi-solid nutrient agar (Tittsler & Sandholzer, 1936).

Both antibiotic treatment before surgery and the preparatory steps can lead to sublethal damage of bacteria and thereby have a negative impact on culture results. Culturing techniques drawing on experience from food microbiology (Wu, 2008) together with culture-independent techniques may help improve diagnosis under these circumstances.

Attention to strict anaerobic precautions during transportation and processing of samples is a key area for improvement of diagnosis of PJIs (Summanen *et al.*, 1993; Tunney *et al.*, 1998, 1999). Based on the current literature, it is tempting to see the isolation rate of *P. acnes* as the best indicator of the proficiency of anaerobic cultures.

Ultimately, one must bear in mind that many bacteria are not cultivable despite all attempts to optimize culture methods; the cultivable fraction from human skin or oral cavity is around 16–20 % of the total diversity and even lower for environmental bacteria (<1 %) (Amann *et al.*, 1995; Kroes *et al.*, 1999; Gao *et al.*, 2007). To complicate matters further, growth of some bacteria may depend on other species and therefore they cannot be isolated in pure culture (Amann *et al.*, 1995).

Acknowledgements

This review was done within the framework of the 'Prosthetic-Related Infection and Pain' (PRIS)-Innovation project. The PRIS-Innovation project is funded by The Danish Agency for Science, Technology and Innovation, Ministry of Science, Technology and Innovation. We are especially indebted to Trine Rolighed Thomsen, Jan Lorenzen and Peter Lüttge Jensen for their constructive criticism.

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Supplementary Table S1. Culture media and incubation periods used for prosthetic joint infections

Studies of prosthetic joint infections with detailed information on culture media and bacterial isolates. Most studies also reported the incubation period. NS, Not stated.

Culture media (supplier)	Conditions	Incubation period (days)	Reference
Blood culture flask, aerobic, paediatric and anaerobic (BACTEC 9240 system; BD and BacT/ALERT; bioMérieux)	Aerobic, anaerobic	5–7	Levine & Evans (2001); Font-Vizcarra *et al.* (2010); van Kats *et al.* (2010)
Thioglycolate broth (unknown)	Aerobic	3–5	Neut *et al.* (2003); Font-Vizcarra *et al.* (2010)
Thioglycolate (BioTrading Benelux)	Aerobic	14	van Kats *et al.* (2010)
Thioglycolate broth (BD)	Aerobic	NS	Levine & Evans (2001)
Tryptone Yeast Glucose (TYG) broth (Oxoid)	Anaerobic	NS	Farrar *et al.* (2007)
Brain heart infusion broth (Oxoid)	Aerobic	14	Schäfer *et al.* (2008); Sendi *et al.* (2010)
Schaedler broth (Oxoid)	Anaerobic	14	Schäfer *et al.* (2008)
Fastidious broth (BD?)	Aerobic	NS	Levine & Evans (2001)
Blood agar (unknown)	Aerobic	2–5	Neut *et al.* (2003); Font-Vizcarra *et al.* (2010)
Blood agar (unknown) +0.5% haemin +0.1% menadione	Aerobic, anaerobic	7	Neut *et al.* (2003)
Hemoline blood agar (bioMérieux)	Aerobic, anaerobic	7	Zeller *et al.* (2007)
Trypticase Soy Broth agar, 5% sheep blood (Oxoid)	Aerobic	14	Schäfer *et al.* (2008)
Sheep blood agar (BD)	Microaerobic, anaerobic, aerobic	4–14	Levine & Evans (2001); Trampuz *et al.* (2006); Piper *et al.* (2009); Font-Vizcarra *et al.* (2010)
Columbia sheep blood agar (bioMérieux)	Aerobic	14	Sendi *et al.* (2010)

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Anaerobic blood agar (BD)	Anaerobic	NS	Levine
Chocolate agar (unknown)	5% CO_2	7	Zeller *et al.* (2007)
Chocolate agar (unknown)	Aerobic	2–4	Neut *et al.* (2003)
Chocolate agar (BD)	Aerobic	Up to 14	Levine & Evans (2001); Schäfer *et al.* (2008); Sendi *et al.* (2010)
Schaedler agar (unknown)	Anaerobic	5	Font-Vizcarra *et al.* (2010)
Schaedler agar (bioMérieux)	Anaerobic	7	Zeller *et al.* (2007)
Schaedler agar +K1, 5% sheep blood (BD) (*P. acnes*)	Anaerobic	14	Schäfer *et al.* (2008)
MacConkey II agar (BD)	Aerobic	4–14	Levine & Evans (2001); Schäfer *et al.* (2008)
Reinforced clostridial agar (Oxoid)	Anaerobic	NS	Farrar *et al.* (2007)
Brucella agar (BD)	Anaerobic	5–14	Neut *et al.* (2003); Sendi *et al.* (2010)
Colistin nalidixic acid (CAN) (BD)	Aerobic	NS	Levine & Evans (2001)
Anaerobic phenyl-ethyl alcohol agar (BD)	Anaerobic	NS	Levine & Evans (2001)
Anaerobic laked kanamycin-vancomycin agar (BD)	Anaerobic	NS	Levine & Evans (2001)
Tryptone Yeast Extract Glucose (TYEG) agar+2 μg furazolidone ml^{-1} (Oxoid)	Anaerobic	7	Ross *et al.* (2003)

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Appendix B. Paper 2

Larsen, L.H., Xu, Y., Simonsen, O., Pedersen, C., Schønheyder, H.C., Thomsen, T.R., and PRIS Study Group (2014). ”All in a box” a concept for optimizing microbiological diagnostic sampling in prosthetic joint infections. BMC Res. Notes 7, 418.

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Larsen *et al. BMC Research Notes* 2014, **7**:418
http://www.biomedcentral.com/1756-0500/7/418

Larsen et al. BMC Research Notes 2014, 7:418
http://www.biomedcentral.com/1756-0500/7/418

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TECHNICAL NOTE Open Access

'All in a box' a concept for optimizing microbiological diagnostic sampling in prosthetic joint infections

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Abstract

Background: Accurate microbial diagnosis is crucial for effective management of prosthetic joint infections. Culturing of multiple intraoperative tissue samples has increased diagnostic accuracy, but new preparatory techniques and molecular methods hold promise of further improvement. The increased complexity of sampling is, however, a tough challenge for surgeons and assistants in the operation theatre, and therefore we devised and tested a new concept of pre-packed boxes with a complete assortment of swabs, vials and additional tools needed in the operating theatre for non-standard samples during a clinical study of prosthetic joint infections.

Findings: The protocol for the clinical study required triplicate samples of joint fluid, periprosthetic tissue, bone tissue, and swabs from the surface of the prosthesis. Separate boxes were prepared for percutaneous joint puncture and surgical revision; the latter included containers for prosthetic components or the entire prosthesis. During a 2-year project period 164 boxes were used by the surgeons, 98 of which contained a complete set of samples. In all, 1508 (89%) of 1685 scheduled samples were received.

Conclusion: With this concept a high level of completeness of sample sets was achieved and thus secured a valid basis for evaluation of new diagnostics. Although enthusiasm for the project may have been a contributing factor, the extended project period suggests that the 'All in a box' concept is equally applicable in routine clinical settings with standardized but complex diagnostic sampling.

Keywords: Prosthesis, Infections, Specimen handling, Specimen types, Transport media

Findings

Background

The microbiological diagnosis plays a crucial role in the effective management of patients with suspected prosthetic joint infection (PJIs) [1]. Diagnostic procedures include percutaneous aspiration of joint fluid as well as revision surgery with retention or removal of prosthetic elements. Chronic foreign body-related infections pose a special challenge because of the diversity of microorganisms involved and their adaption to a subdued lifestyle associated with formation of biofilms. Culturing of multiple samples has been shown to increase diagnostic accuracy, and there is growing evidence to support the utility of new preparatory techniques and molecular methods [2-4].

As a direct consequence of this development the number and types of samples wanted from the surgical field are increasing, and the sampling procedure thereby becomes more cumbersome for the surgeon. Even with assistance from a skilled nurse on the floor of the operating theatre important samples can be missed or deposited in an unsuitable transport medium, and the diagnostic accuracy can thereby be compromised [2,4-6].

Within an ongoing research project comprising patients with a painful prosthetic joint ('Prosthesis: Related Infection and Pain' (PRIS), www.joint-prosthesis-infection-pain.dk) we have addressed this issue by designing pre-packed boxes containing disposable scalpels and forceps, swaps, transport

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vials, and labels needed for sampling during the surgical procedure. Additional boxes were made available for samples of synovial fluid obtained by percutaneous joint aspiration. Our primary aim was to overcome the variation in sampling technique within and between surgical teams and across difference hospitals, which otherwise might affect the validity of our clinical study. Our belief was that streamlining sampling procedures would maximize the completeness of sample sets. We here present the results from a 2-year project period.

All in a box

We applied the 'All in a box' concept to two surgical procedures and report the completeness of sampling within a prospective cohort of patients undergoing revision surgery.

The concept was developed jointly by orthopaedic surgeons, molecular biologists, and clinical microbiologists within the framework of the PRIS project. The project was approved by the Regional Committee on Health Research Ethics (June 2011; ref. no. N-20110022). Informed oral and written consent was obtained from each patient.

The sample repertoire was supplementary to five intraoperative soft tissue biopsies obtained according to the Kamme and Lindberg principle [7]. For revisions the non-standard samples comprised joint fluid, intraoperative soft tissue and bone biopsies, swabs from the surface of the prosthesis *in situ*, and prosthetic components or the entire prosthesis. Diagnostic methods included bacteriological culture, 16S *rDNA* gene amplification followed by amplicon sequencing, and fluorescence *in situ* hybridization (FISH). Thus, samples were obtained in triplicate except for the prosthesis itself or prosthetic components. Each sample was handled separately with disposable utensils in order to minimize cross-contamination [7,8] and thus allow valid comparison of different sample types and analyses.

The two types of boxes are presented in Figure 1 and Table 1. The sample collecting kit for revision surgery consisted of scalpels and forceps, and a special needle for a bone biopsy (Vertebroplasty Needle, Synthes, West Chester PA, USA). Sample tubes were colour coded according to sample type. For collection of biopsies, tubes with a broad neck were chosen to facilitate handling in the operating theatre as well as in the laboratories. A sterile container of an appropriate size for the prosthetic component was included for revision surgery. The only item not included in the pre-packed boxes was a blood culture vial for synovial fluid due to its limited shelf life.

From the surgical theatre the boxes were transported at ambient temperature to the Department of Clinical Microbiology and processed within 24 h. Most samples were processed within 2 h after removal of the prosthesis, whereas samples from acute surgery undertaken out of hours were kept at 4°C overnight except for the blood culture vial that was held at room temperature. When delivered to the lab, samples for molecular analysis were subjected to vigorous agitation (vortexing for 30 sec) and stored immediately at −80°C until batch wise processing and analysis. For microbiological culture components of the prosthesis (covered with PBS-buffer, pH7.4) were vortexed and sonicated (summarised in [2]). Bone biopsies were treated similarly before culturing. The joint fluid, tissue biopsies, and the prosthesis swab were cultured without pre-processing. All sample types were cultured aerobically and anaerobically for 14 days with subcultivation from enrichment broth after 6 days for positive samples and after 10 days for negative samples.

All surgeons undertaking revision surgery were informed about the box design and agreed to the concept. The implementation benefitted further from liaison with the nurses assisting in the operating theatre. Of note, joint punctures took place in both ambulatory and in-hospital

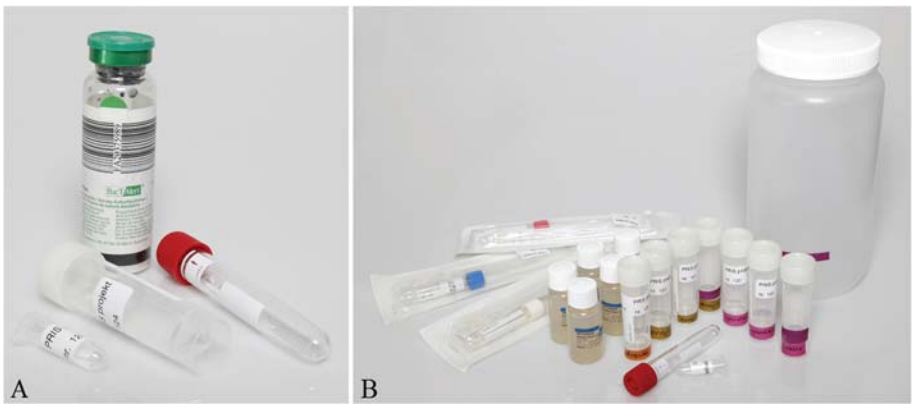

Figure 1 Sample boxes for joint puncture (A) and revision surgery (B). A: Joint fluid is both inoculated directly into a blood culture flask (BacT/Alert, bioMérieux, Marcy l'Etoil, France) and submitted for extensive culture examination and molecular diagnostics. **B**: Sample tubes are colour coded in the revision surgery box in order to assist the operation staff in achieving complete sample sets. Sample tubes had a broad neck in order to facilitate the deposition of the sample.

Table 1 Boxes' design and transport media

	Specimen type	Bacteriological culture	*16S rDNA* sequencing	FISH
Joint puncture	Synovial fluid	15 mL tube (empty)*	Tube B	Tube C
		Blood culture vial		
Revision surgery	Synovial fluid	15 mL tube (empty)	Tube B	Tube C
	Soft tissue	Tube A	Tube D	Tube C
		Vials with Stuart transport medium (x5)*		
	Bone biopsies	Tube A	Tube D	Tube C
	Swabs from prosthesis***	Tube A	Tube D	Tube C
		(ESwab)		
	Prosthesis (components or in toto)	Empty container**	Tube B**	Tube C**

Tube A: Modified Amies medium (Copan). Tube B: 2 mL tube with 60% glycerol in DEPC water, targeting a final concentration ≥10% glycerol including sample; estimated final concentration ~15-20%. Tube C: CyMol® (Copan). Tube D: Modified Amies medium with 20% glycerol (Copan). The transport media were 1] modified Amies medium for direct culture, 2] CyMol® for the preservation of nucleic acids for FISH, and 3] modified Amies medium with 20% glycerol (estimated final glycerol content ~10% incl. sample) for storage of whole bacterial cells at −80°C for subsequent molecular analyses (Copan). Stuart medium (SSI Diagnostica, Copenhagen Denmark) was used for biopsies of periprosthetic soft tissue obtained according to Kamme and Lindberg [7] and handled accordingly since the 1980's.

*Samples taken routinely during surgical revision.

**The processing of prosthetic components took place in the laboratory (summarized in [2]). ***Swabs used to prosthetic scraping *in situ* for culture and 16S *rDNA* gene sequencing were CLASSIQSwab and for FISH (CyMol®) a FLOQSwab (Copan).

settings, and they were less rigidly standardized compared with revisions.

'Proof of concept'

The scheduled number of samples was four for percutaneous joint aspiration (box A) and 13 for revision surgery (box B) (Table 1). From December 2011 through February 2014 we obtained 98 boxes with a complete sample set out of 164 consecutive boxes (box A: 25/42 (60%); box B: 73/122 (60%)). In all, 1508 of 1685 scheduled samples were obtained (overall completeness 89%). The main reasons for missing samples were deviations from the pre-planned surgical procedure for clinical reasons or absence of a trained assistant. In 8 cases the sample set in box B was incomplete as a consequence of acute surgery (69 of 104 scheduled samples (66%)).

Experience and perspective

We find the 'All in a box' to be a promising logistic concept for obtaining multiple samples as part of surgical procedures. The concept may be applicable not only to the diagnosis of PJIs but also to other diagnostic procedures and would be well suited especially in circumstances where limited amounts of sample material must be shared between several diagnostic tests and the use of a correct transport medium plays an important role for the performance of the diagnostic test. An obvious addition to the different microbiological tests in this clinical study would be tissue samples for histopathology. Despite the complex intraoperative sampling procedure the 'All in a box' concept provided an overall completeness around 90% in a research project involving several orthopaedic surgeons, numerous nurses, and different hospital premises.

The concept should also be applicable to other complex sampling procedures utilizing a standardized panel of diagnostic sample types and thus has a potential for time saving and optimization in different diagnostic settings. Although we ascribe the high level of completeness in our study to the 'All in a box' concept, it must be acknowledged that enthusiasm surrounding the research project may also have been involved. Still, the positive attitude often withers when procedures are complicated and involve many surgeons and nurses, but it was our impression that the box logistics helped to maintain the spirit in this case. A drawback to the concept was the time consumed by the meticulous preparation of the boxes, a task which can be managed within the framework of a scientific project, but may be difficult to tackle on a routine basis in hospitals and clinics. We estimate the cost of materials for box A to be 130 €, and the full diagnostic work-up of samples in this box may amount to 1075 € including extensive 16S *rDNA* sequencing. Implementation of only the most effective diagnostic modalities may help to decrease these costs. Moreover, the concept could be of interest to providers of diagnostic utensils and could also be instrumental in implementing standardized sampling procedures eventually based on international guidelines.

Competing interests

The authors declare that they have no competing interest.
The transport medium 'Modified Amies media with 20% glycerol' was developed in cooperation between the authors and Copan Italia S.P.A. (Brescia, Italy). All transport media were provided by Copan, if not otherwise indicated. Copan did not influence on the manuscript and the authors have no financial interests to declare.

Authors' contributions

OS, TRT and HCS designed and developed the first versions of the boxes. After inputs from YX and CP the first pilot study was conducted with OS and

CP as surgeons [3]. LHL, YX, TRT, HCS and OS optimized the boxes for the clinical study. LHL and YX were responsible for the overall management of the boxes. LHL prepared the first draft of the manuscript and all authors read and approved the final manuscript.

Acknowledgments
This technical note was prepared within the framework of the 'Prosthetic-Related Infection and Pain' (PRIS) - Innovation project, www.joint-prosthesis-infection-pain.dk. The study was supported by a grant for the PRIS Innovation Consortium from The Danish Council for Technology and Innovation (no. 09–052174).
Thanks to the PRIS Study Group and especially to Poul T. Nielsen, Jess T. Riis, Andreas Kappel, Mogens B. Jørgensen, Mogens B. Laursen, Vesal Khalid, Camilla R. Rethleff, Ulla Hornum, Dorthe Kvorning, Lasse Lengsø, Susanne Bielidt, Lena Mortensen, Mette Mølvadgaard, Jan Lorenzen and the staff from the Department of Clinical Microbiology and the Orthopaedics Departments for their practical contributions to the sampling.
Special thanks also to Santina Castriciano (Copan Italia A.S.P.) for her unfailing interest in our project.

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Received: 7 October 2013 Accepted: 26 June 2014
Published: 3 July 2014

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doi:10.1186/1756-0500-7-418
Cite this article as: Larsen *et al.*: 'All in a box' a concept for optimizing microbiological diagnostic sampling in prosthetic joint infections. *BMC Research Notes* 2014 **7**:418.

Appendix C. Paper 3

Larsen, L.H., Khalid, V., Xu, Y., Thomsen, T.R., Schønheyder, H.C. and the PRIS Study Group.
Diagnostic value of culture and 16S r*RNA* sequencing in patients undergoing revision surgery for infection of a hip or knee arthroplasty.
Draft

Appendix D. Paper 4

Larsen, L.H. Xu, Y, Nielsen, K.L., Schønheyder, H.C., and Thomsen, T.R.
In vivo gene expression in a *Staphylococcus aureus* biofilm infection model in guinea pigs: Impact of antibiotic treatment with moxifloxacin.
Draft

Appendix E. Poster Larsen *et al.* 2013

Larsen, L. H., Frost, M., Aleksyniene, R., Kappel, A., Khalid, V., Schønheyder, H. C., Thomsen, T. R. & Group, P. S. (2013). Chronic prosthesis joint infection - Case report. In *3th Eurobiofilms*. Presented at the Eurobiofilms 2013.

Chronic prosthesis joint infection

Case report

Lone Heimann Larsen[1,4], Majbritt Frost[2], Ramune Aleksyniene[2], Andreas Kappel[3], Vesal Khalid[3], Henrik C. Schønheyder[1], Trine Rolighed Thomsen[4,5] and the PRIS study group

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1994
Primary prosthesis: total knee replacement (TKA) with an uncemented prosthesis, followed shortly by an insertion of a cemented patella component.

2005
Contact to the hospital with severe pain, instable TKA. Diagnostic imaging revealed synovitis deemed to be related to ongoing rheumatologic disease. A revision was carried with replacement of both the tibia and femur components combined with synovectomy.
The patella component was retained. Peroperative specimens revealed coagulase-negative staphylococci in 3 out of 5 synovial biopsies and the patient was put on treatment with antibiotics.

2006
After the revision the patient had recurrent stress-related pain in the proximal tibia, swelling of the knee and a slight, but persistent elevation of inflammatory markers. Repeated joint aspirations were negative on standard (aerobic &anaerobic culture. The patient was treated with a brief course of glucocorticosteroids.

2012
The patient had persistent stress-related pain in the proximal tibia and swelling of the knee. The patient was included in the PRIS project (www.joint-prosthesis-infection-pain.dk) and advanced diagnostic hybrid imaging (bone scan, dual leukocyte/bone marrow SPECT-CT and PET-CT) showed a 'hotspot' at the interface between the lateral tibia plateau and the prosthesis. The TKA was removed and submitted to extensive microbiological diagnostics that included ultrasonication of the prosthetic component. The microbiology showed *Staphylococcus epidermidis* with an antibiogram similar to that reported in 2005, making it very likely that a chronic biofilm prosthesis infection had persistent for the last 7 years. The patient was treated with antibiotics and the infections parameter was normalised for the first time since 1994.

Day 0
Revision surgery
Removal of the prosthesis, spacer were inserted

Day 6
Cultures of sonication buffer from the prosthesis components revealed *S. epidermidis* app. $4.5x10^4$ CFU/prosthesis with an antibiogram distinctly similar to that reported in 2005.

Day 14
Enrichment broth culture of synovial tissue and joint fluid were positive for *S. epidermidis* (prosthesis, tissue, joint fluid) and *Propionibacterium acnes* (prosthesis only; CFUs were insignificant).

2 Months
A new prosthesis was inserted. Inflammatory markers were normalised for the first time since 1994. If microbiological cultures had not been incubated for 14 days, there is a definite risk that the *S. epidermidis* infection had been missed again, and the new prosthesis would have been inserted before the infection might have been cleared.

Acknowledgement
This study is part of the Danish ´Prosthetic-Related Infection and Pain´ Innovation Project (Danish acronym PRIS), supported by a grant from the Danish Agency for Science, Technology and Innovation, Danish Ministry of Science, Innovation and Higher Education.
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Appendix F. Poster: Larsen *et al.* 2014b

Larsen, L. H., Xu, Y., Pedersen, M. S., Schønheyder, H. C. & Thomsen, T. R. (2014b). Long-term storage of clinical samples in CyMol® medium for PNA-FISH® and culturing from the eSwab™ system. In *24th ECCMID*. Presented at the 24th European Congress of Clinical Microbiology and Infectious Diseases, ECCMID 2014.

P606

Long-term storage of clinical samples in CyMol® medium for PNA- FISH® and culturing from the eSwab™ system

Lone Heimann Larsen[1,2], Yijuan Xu[2], Malene S. Pedersen[2], Henrik C. Schønheyder[1,3], Trine Rolighed Thomsen[2,4] and the PRIS study group

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Objectives: The diversity of bacteria reported in foreign body infections is steadily growing, and culture-independent methods have become a valuable supplement to established culture methods. Therefore, sampling and preservation of specimens for molecular analysis have become an important issue. We report here experience gained with different specimen types obtained in parallel both pre- and peroperatively in patients enrolled prospectively in a clinical study of prosthesis-related problems (www.joint-prosthesis-infection-pain.dk).

Methods: Parallel sampling for both culture-dependent and -independent analyses were done over a period of two years. Specimens included tissue biopsies, bone biopsies, joint fluid, and eSwabs™ (Copan, Italy) taken from the prosthesis *in situ*, as well as sonication fluid (if prosthesis components were removed). Transfer to the laboratory was direct (ambient temperature) and storage temperature for culture specimens was +4°C (max 24 h), and -80°C for specimens for culture-independent methods (until batchwise analysis). Specimens for peptide nucleic acid-fluorescence *in situ* hybridization (PNA-FISH) analysis were stored in CyMol® medium (Copan, Italy) for up to one year. Direct visualization of microorganisms followed a previously published PNA-FISH® (AdvanDx, USA) protocol except for a substitution of the fixation step with filtration of 200 µL sample through a 0.22 µm white polycarbonate filter (prewashed with dH_2O, GE Water & Process Technologies, USA) in order to fix and concentrate the sample.

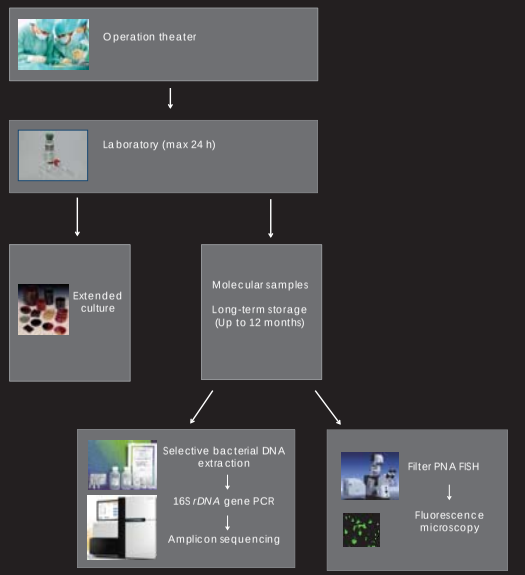
Results: With broad range and specific PNA-FISH® probes, we demonstrated bacteria with a bright signal and morphology comparable to the isolates obtained by culture of parallel specimens within 24 h. The detection limit for PNA-FISH® were >10^2 CFU/mL (estimated from the colony forming units cultured, updated from abstract). With the eSwab™ system we detected a broad range of Gram-positive taxa including *Staphylococcus* spp., *Streptococcus* spp., *Enterococcus* spp., and *Corynebacterium* spp. by culture and 16S rRNA gene amplicon sequencing (table 1).

Conclusion: The use of the CyMol® medium made it possible to preserve samples at -80°C for study by PNA-FISH® for at least 12 months and we expect storage for even longer periods to be feasible. We estimated the effective detection limit to be in the order of >10^2 CFU/mL (updated from the abstract). Both the morphology and intensity of staining with nucleic acid and PNA probes were distinct. The eSwab™ was a convenient system for documenting a broad range of bacterial pathogens associated with foreign body infections.

	Direct culturing	16S *rDNA* sequencing
	eSwab™ in modified Amies medium	eSwab™ stored in modified Amies medium with 20% glycerol at -80 °C
Staphylococcus aureus	+	+
Staphylococcus epidermidis	+	+
Staphylococcus lugdunensis	+	+
Staphylococcus caprae	+	+
Enterococcus faecalis	+	
Streptococcus dysgalactiae	+	+
Streptococcus agalactiae	+	+
Propionibacterium acnes	+	+
Corynebacterium jeikeium	+	+
Corynebacterium striatum	+	
Escherichia coli		+

Table 1: Bacterial species recovered by eSwab™ (Copan, Italia). eSwabs™ used for culturing were transported in modified Amies medium and cultured within 24 h. eSwabs™ for sequencing were stored at -80 °C in modified Amies medium with 20% glycerol until analysed batchwise.

Flow diagram



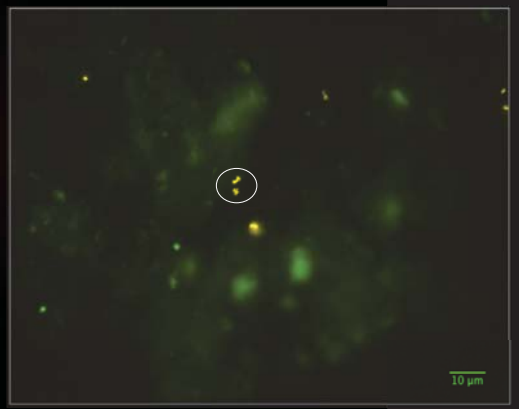


Figure 1: Filter PNA-FISH® on sonication fluid from hip prosthetic component. Universal bacterial probe (BacUni; green, *Enterococcus faecalis*; red. The culture and sequencing were positive for *E. faecalis*. The PNA-FISH confirmed the culturing and sequencing results illustrating yellow bacteria in clusters (eg. circled).

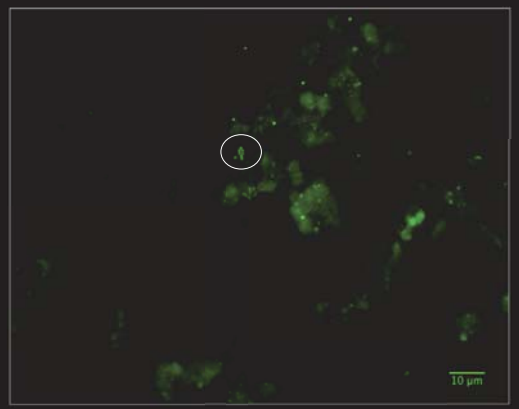


Figure 2: Filter PNA-FISH® on sonication fluid from knee prosthetic component. *Staphylococcus aureus*; green. The culture and sequencing were positive for *S. aureus*. The PNA-FISH confirmed the culturing and sequencing results illustrating green bacteria in clusters (eg. circled).

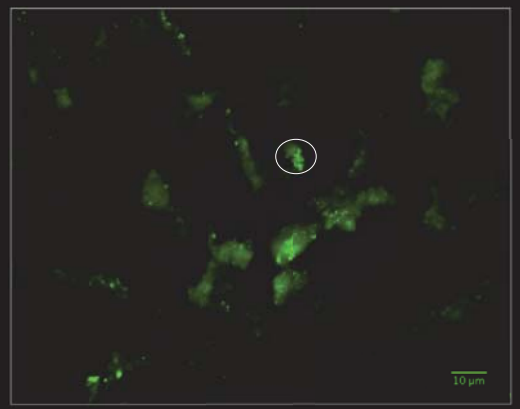


Figure 3: Filter PNA-FISH® on sonication fluid from knee prosthetic component (same case as figure 2). *Staphylococcus aureus*; green. The culture and sequencing were positive for *S. aureus*. The PNA-FISH confirmed the culturing and sequencing results illustrating green bacteria in clusters (eg. circled).

Acknowledgement
This study is part of the Danish 'Prosthetic-Related Infection and Pain' Innovation Project (Danish acronym PRIS) and supported by The Danish Council for Technology and Innovation (no. 09-052174). **www.joint-prosthesis-infection-pain.dk**

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ISSN (online): 2246-1248
ISBN (online): 978-87-7112-777-5

AALBORG UNIVERSITY PRESS